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An Investigation into the Postnatal Development of the Enteric Nervous System

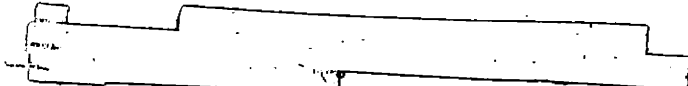
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Abbreviations

ABC	Avidin-biotinylated complex
ABDS	Antibody diluting solution
Ach	Acetylcholine
AChE	Acetylcholine esterase
Anova	Analysis of variance
ART	Artemin
ATP	Adenosine tri-phosphate
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BOM	Bombesin
BSA	Bovine serum albumin
Bss	Balanced salt solution
CALRET	Calretinin
CCK	Cholecystokinin
CGRP	Calcitonin gene related peptide
ChAT	Choline acetyltransferase
CNTF	Ciliary neurotrophic factor
DAB	Diaminobenzine
D β H	Dopamine β hydroxylase
dPBS	Dulbecco's phosphate buffered saline
DYN	Dynorphin
E	Embryonic day
ENK	Enkephalin
eNOS	Endothelial nitric oxide synthase
ENS	Enteric nervous system
EM	Electron microscopy

FCS	Fetal calf serum
γ GABA	γ amino butyric acid
GAL	Galanin
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary associated protein
GI	Gastrointestinal
GLU	Glutamate
GRP	Gastrin related peptide
GFR	Glial cell line-derived factor receptor
HBSS	Hank's balanced salt solution
5-HT	5-Hydroxytryptamine
ICC	Interstitial cells of Cajal
IHC	Immunohistochemistry
IL-6	Interleukin-6
IN	Alpha inter-nexin
INOS	Inducible nitric oxide
KDa	Kilo Dalton
LBP110	Laminin binding protein (110KDa)
LIF	Leukaemia inhibitory factor
MASH-1	Mammalian homologue of <i>achaete-scute</i>
MP	Myenteric plexus
N	Number
NA	Noradrenaline
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NANC	Non-adrenergic non-cholinergic
NC-1	Neural crest -1

NCB	Nerve cell body
NGF	Nerve growth factor
NGS	Normal goat serum
NFP	Neurofilament protein
NK-1	Neurokinin -1
NMU	Neuromedin
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
NPY	Neuropeptide Y
NT	Neurotrophin
NTN	Neurturin
P	Significance level
PACAP	Pituitary adenylyl-cyclase-activating peptide
PER	Peripherin
PGP 9.5	Protein gene product 9.5
PSP	Persephin
RET	Re-arranged during transfection
Rpm	Revolutions per minute
SA	Sympathoadrenal
Se	Standard error of the mean
SI	Small intestine
SMP	Submucous plexus
SOM	Somatostatin
SUBS P	Substance P
TGFβ	Transforming growth factor beta
TRK	Tyrosine receptor kinase
VIP	Vasoactive intestinal polypeptide

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Abstract

The postnatal development of the myenteric plexus from the rat ileum was studied using a variety of techniques. Immunohistochemistry of wholemount preparations for the neural marker PGP 9.5, revealed changes in the density of myenteric neurons during postnatal development. A significant increase in neuronal numbers was observed between 7 and 14 days. NADPH diaphorase positive neurons were among those that increased in number during this time. Total neuronal numbers decreased significantly between 14 and 21 days, possibly indicating a period of naturally occurring cell death. There was no change in the number of NADPH diaphorase positive neurons during this time. A second significant increase in neuronal numbers was observed between 21 and 28 days. Total neuronal numbers appeared to stabilise at 35 days. Ultrastructural analysis revealed early postnatal (7 day) neuron and glial profiles to be relatively mature and exhibit a similar variety of shapes as seen in the adult rat and guinea pig. The basal lamina that surrounds most adult myenteric ganglia, was found to be developmentally acquired. The number of smooth muscle cells increased between 7 and 21 days, then numbers stabilised and the thickness of the smooth muscle increased. Possible stimuli for the observed changes in the myenteric plexus include GDNF, a neurotrophic factor that is expressed in postnatal intestinal muscle. GDNF was found to stimulate an increase in the number of myenteric neurons and neurite outgrowth in culture. GDNF did not however, influence other aspects of myenteric neuronal growth such as somal size, suggesting that other molecules may also be involved in the postnatal development of myenteric neurons. In summary, the work presented in this thesis has highlighted the plasticity of the postnatal enteric nervous system and the importance of the postnatal period in establishing the adult pattern of innervation and diversity of phenotypes.

CHAPTER 1

INTRODUCTION

General Introduction

The enteric nervous system (ENS) regulates gut function and extends the length of the gastrointestinal (GI) tract. It is a complex meshwork of sensory, motor and inter-neurons and this complexity is reflected in the large number of neurons present in the ENS and by their great phenotypic diversity (see Costa et al. 1987; Costa et al. 1992;). Enteric neurons are organised into two interconnected ganglionated plexuses; the myenteric and the submucosal plexus, which are embedded in the gut wall, and can act largely independently of the central nervous system (see Wood 1981; Gershon et al. 1994). The organisation of enteric ganglia is unlike that of other autonomic ganglia and although there are regional variations, each enteric plexus has a characteristic arrangement (Costa et al. 1987; Furness et al. 1994). Enteric ganglia also exhibit several characteristics of central nervous system tissue (Gabella 1979; Jessen and Mirsky 1980; Jessen and Mirsky 1983; Jessen et al. 1983a; Furness et al. 1994).

Functions of the enteric nervous system include the control of intestinal motility, secretion and blood flow. The myenteric (or Auerbach's) is the outermost plexus and lies between the longitudinal and circular muscle layers of the muscularis externa. It is the larger plexus and is primarily concerned with control of motility within and along the gastrointestinal tract. The submucous plexus is located within the submucosal layer and is primarily concerned with mucosal functions, such as the regulation of bloodflow and secretions. Enteric neurons form a diverse population with a variety of neurotransmitters and projections. Subpopulations project to all layers of the gut wall where they innervate smooth muscle, mucosal epithelial cells, blood vessels and other enteric neurons. Subpopulations of enteric neurons also project to the liver and pancreas.

Several criteria have been employed to categorise or “code” subpopulations of enteric neurons (Stach, 1989 and Wood, 1994). These include; morphology (for example Dogiel 1895; Dogiel 1896; Dogiel 1899; see Furness et al. 1988), histochemistry and neurotransmitter phenotype (See Bornstein and Furness 1992; Costa et al. 1992; Costa et al. 1987; (Gershon et al. 1994) and electrophysiology, (Bornstein et al. 1994). It is clear that under each classification system there are exceptions, thus a complete coding of enteric neurons is probably impossible (see Stach 1989; Wood 1994).

Knowledge of how the enteric nervous system develops to reach such complexity is necessary to aid the understanding of congenital and acquired lesions, as well the development of potential therapies for such diseases. Lesions of the enteric nervous system result in malformations of the GI tract that may be severe, or even fatal (for example Beck and Podolsky 1999; Collins et al. 1997; Hanauer 1996; Kapur 1993; Kapur et al. 1996; Krishnamurthy and Schuffler 1987). Embryological studies have shown that the environment of the developing gut plays an important role in determination of the phenotype of enteric neurons (for example Rothman et al. 1986; See et al. 1990; Nishijima et al. 1990; Rothman et al. 1990; LeDouarin and Dulac 1992; LeDouarin and Dupin 1993; Bronner-Fraser 1993; Chalazonitis et al. 1994; Natarajan et al. 1999). However, as the enteric nervous system is extremely complex and largely inaccessible, relatively little is known about the factors controlling ganglion formation, neuronal growth and differentiation and target cell innervation during pre- and postnatal development.

Postnatal development of the enteric nervous system and achievement of the adult level of maturity is not well characterised, although neuronal differentiation has been shown to be ongoing during the first month of postnatal life (Pham et al. 1991; Faussonne-Pellegrini et al. 1996; Matini et al. 1997; Vannucchi and Faussonne-Pellegrini 1996) indicating that at birth enteric neurons are relatively immature with regard to this aspect. The work in this thesis was undertaken to study the development of the rat enteric nervous system in detail during the postnatal period using a variety of techniques.

Following the Introductory and Methods chapters (chapters 1 and 2), the observations made in this study are presented in 4 main results chapters (Chapters 3 – 6) and the significance of these observations, both to current knowledge and to future research in this area are described in the final chapter, the General Discussion.

Chapter 3 details work performed to study possible changes in the density, appearance and distribution of myenteric neurons during postnatal development, using immunohistochemistry for a neural marker (PGP 9.5) on wholemount stretch preparations of ileum. Details of small intestinal growth are also presented in this chapter. A detailed ultrastructural study of myenteric neurons, glial cells and surrounding smooth muscle during the postnatal period is presented in Chapter 4. The postnatal development of the nitric oxide synthesising subpopulation of myenteric neurons was studied using the NADPH-diaphorase histochemical method on wholemount preparations of ileum and the results are presented in chapter 5.

The investigation discussed in Chapters 3 - 5 revealed changes in the myenteric plexus during the first month of postnatal life. The factors stimulating the observed changes are unknown, however an increase in neuronal density could occur as the result of the differentiation of precursor cells.

Several factors have been shown to stimulate the differentiation of enteric precursors and enteric neurons *in vitro*. One factor that has shown to be crucial for the prenatal development of the enteric nervous system is glial cell-line derived neurotrophic factor (GDNF). Potential trophic effects of this factor in the postnatal enteric nervous system were investigated on myenteric neurons grown in a dissociated tissue culture system. The results of this investigation are presented and discussed in chapter 6.

The remainder of the present chapter will describe previous studies of enteric morphology, ultrastructure, neurochemistry and development.

Historical Introduction

This section describes some of the anatomical, neurochemical and functional properties of the enteric nervous system, in order to illustrate the complexity and importance of this system. Several attempts have been made to quantify and categorise enteric neurons in the adult intestine, both at the light and electron microscope level and a review of relevant literature is presented. Studies of enteric nervous system development are discussed and the possible roles of neurotrophic factors in this process are described. An account of some of the different techniques that have been used to study the developing and adult enteric nervous system, together with some of their advantages and disadvantages are also presented. Details of the many physiological and pharmacological studies that have been performed on the enteric nervous system and gastrointestinal tract are not included, although a brief summary of findings, as well as references to comprehensive reviews and original papers are given where appropriate.

1.1 General anatomy of the mammalian gastrointestinal tract and enteric nervous system

The gastrointestinal (GI) tract consists of three major, anatomically distinct regions; the stomach, small intestine and large intestine. The duodenum, jejunum and the ileum together form the small intestine which is involved mainly with digestion and absorption of ingested foodstuffs. The large intestine is comprised of the cecum, colon, rectum and anal canal and is predominantly involved with the compaction, storage and movement of waste products. In appearance, the small intestine is generally longer and narrower than the large intestine. Externally, the small intestine has a smooth appearance and the large intestine appears folded.

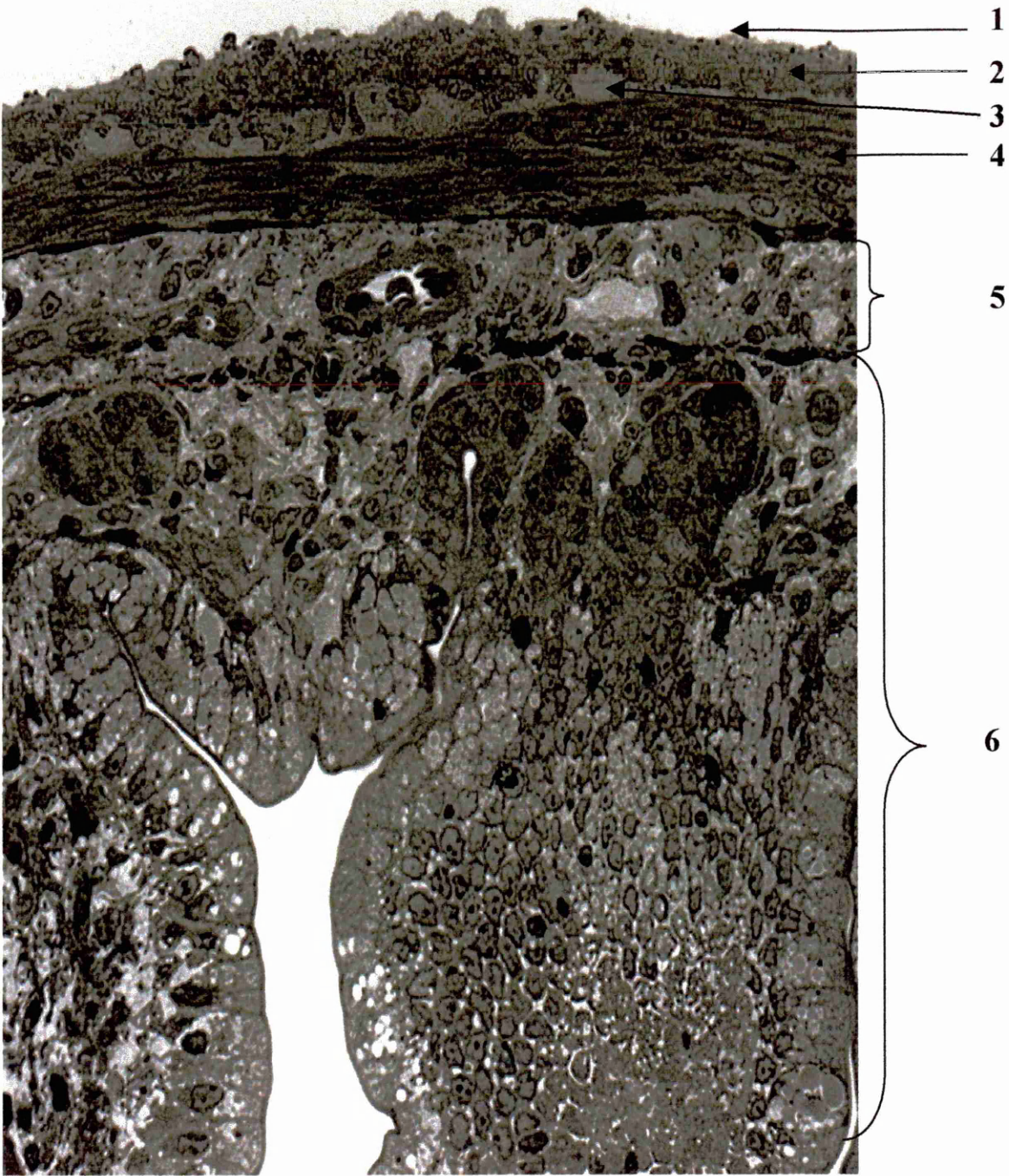
In the large intestines of some mammals (e.g. the guinea pig), parts of the longitudinal muscle are concentrated into three bands called *taenia coli*, which draw in the large intestine and give the appearance of pouches called haustra.

Developmentally, the foregut gives rise to the pharynx, the oesophagus, stomach and the upper half of the duodenum. The midgut gives rise to the remaining half of the duodenum, jejunum, ileum, cecum, appendix, ascending colon and two-thirds of the transverse colon. The hindgut gives rise to the remaining third of the transverse colon, descending colon, sigmoid colon and the rectum (see Thompson and Keelan 1986). In the human gut, the boundaries of the foregut, midgut and hindgut are taken as corresponding to the territories of the three arteries that supply the developing abdominal tube (see Larsen 1993).

The gastrointestinal (GI) tract has a number of functions, including the mixing and propulsion of ingested materials, as well as secretion of digestive juices and absorption of nutrients and water. The appearance of the GI tract changes along its length in relation to changes in function, although the general organisation is similar in all regions. Figure 1a shows a semithin section through rat ileum and shows the typical arrangement of intestinal layers in transverse section.

The serosa is a layer of epithelial cells that is continuous with the connective tissue of the mesentery and is the outermost covering of the GI tract. The blood vessels and extrinsic nerve fibres that supply the intestine reach the gut wall via the mesentery. Beneath the serosa lies the muscularis externa, which consists of two smooth muscle layers, the longitudinal and circular muscle layers and the myenteric plexus.

Figure 1a An adult rat intestine in section



- | | |
|---|---------------------|
| 1 | Serosa |
| 2 | Longitudinal muscle |
| 3 | Myenteric plexus |
| 4 | Circular muscle |
| 5 | Submucosa |
| 6 | Mucosa |

100µm

A transverse semithin (1µm) section through a portion of adult rat ileum stained with Toluidine blue. For preparation details see section 2.4.2. The circular and longitudinal smooth muscle layers are easily distinguished and the myenteric plexus is prominent. The submucous plexus is difficult to discern on this section. Although the gut exhibits regional differences in the wall thickness, the basic plan is the same along the length of the tract.

The long axes of the longitudinal smooth muscle cells run parallel to the length of the GI tract, whereas the long axes of circular muscle cells run around the circumference of the GI tract. Enteric smooth muscle is arranged in bundles and has a high level of connectivity which is both anatomical (i.e. branching of muscle cells) and electrical (e.g. gap junctions), allowing muscle cells contract in unison when stimulated see (Gabella 1992; Maudlej and Hanani 1992). The myenteric plexus is a meshwork of ganglia that lie between the circular and longitudinal muscle. Myenteric neurons project to other enteric ganglia and gut wall layers, as well as to enteric smooth muscle, although contacts between enteric neurons and muscle fibres have a varicose appearance and lack the specialised structure of neuromuscular junctions observed in the somatic division of the peripheral nervous system.

Beneath the circular muscle lies the submucosa, which is a connective tissue layer containing nerve fibres and the major intestinal blood vessels, and the smaller of the enteric nerve plexuses, the submucous plexus. Between the submucosa and the gut lumen lies the mucosa, which is comprised of a thin smooth muscle layer (the muscularis mucosa), a layer of connective tissue (lamina propria) and a surface layer of epithelium. The epithelium is in direct contact with the luminal contents and is thrown into folds in order to increase surface area available for absorption.

1.2 Functions of the mammalian enteric nervous system

Enteric ganglia are organised into the myenteric (or Auerbach's) and the submucosal (or Meissner's) plexuses, each with a characteristic arrangement of enteric ganglia, as defined by ganglion size, shape and distance between individual ganglia (interganglionic distance).

Enteric ganglia are highly interconnected, with complex projections, although the myenteric plexus is primarily concerned with the neural control of ingesta movement both within and along the gastrointestinal tract. The submucous plexus is primarily concerned with the regulation of secretion and absorption within the gastrointestinal tract and is generally treated as a single entity but probably consists of two separate but highly interconnected plexuses. Those plexuses are the plexus of Meissner, which is nearest to the mucosa and the plexus of Schabadasch, which is outermost and nearest to the circular muscle. As the connections between the two are extensive it was thought unnecessary to refer to them separately but recent studies have shown anatomical, electrophysiological and neurochemical differences between the two submucosal plexuses which may indicate functional differences (see Gershon et al. 1994).

Initially the enteric nervous system was thought to be a simple parasympathetic relay station (see Costa et al. 1987 for review). Pharmacological, lesion and physiological studies however, have revealed a far more complex situation and enteric neurons have been shown to be diverse both in both function and phenotype. The enteric nervous system contains sensory, motor and inter-neurons (see Furness and Costa 1980 and section 1.5).

Enteric sensory neurons detect distension of the gut wall as well as changes in the chemical composition of luminal contents. Neurons with a specific morphology (Dogiel type II morphology, see section 1.3.2) have been proposed as sensory neurons on the basis of their projections and observations that they respond to serosal distension or mucosal distortion (see Gershon et al. 1994). Enteric motor neurons stimulate contraction and relaxation of smooth muscle, as well as mucosal secretion (secretomotor).

Neurons with Dogiel type I morphology have been proposed to be motor neurons on the basis of their projections (see Gershon et al. 1994). Enteric interneurons are diverse in morphology and project both between and within the enteric plexuses and are thought to have an integrative function (see Costa et al. 1987; Gershon et al. 1994). Each functional type of enteric neuron contains subpopulations of both short and long range projecting neurons, which exert excitatory and inhibitory actions through several combinations of neurotransmitters.

1.3 Morphological studies of the enteric nervous system

1.3.1 Neuronal Numbers

Examination of possible changes in the enteric nervous system during development, experimental manipulation, disease states and ageing requires reliable staining methods that will identify all enteric neurons, regardless of their subtype. Such neuron-specific staining methods are also required to assess the proportions of neurons within individual subpopulations. Several methods have been employed to assess total numbers of neurons within enteric plexuses, with varying degrees of success.

Myenteric neurons have been visualised in various species and gut regions using stretch preparations of the myenteric plexus. Histochemical stains such as toluidine blue, methylene blue, thionine, giesma and silver stains (see Costa et al. 1987) as well as Cuprolinic blue (Heincke et al. 1987; Karaosmanoglu et al. 1996) have been shown to be taken up specifically by neurons in these preparations. However, not all neurons are labelled by these methods. A histochemical method of identifying the dehydrogenase NADH-diaphorase has been used to quantify total neuronal numbers, based on the assumption that the majority if not all myenteric neurons can be detected using this method.

Gabella (1987) and Santer et al. (1988), amongst others, utilised this method to estimate total numbers of neurons within the myenteric plexus; Santer et al. (1988) estimated that there are 8169 ± 413 neurons/cm² in the myenteric plexus of the 6 month rat ileum using this technique.

Subsequently, the assumption that all myenteric neurons can be detected by NADH diaphorase histochemistry has been shown to be incorrect and it has been demonstrated that not all enteric neurons, at least in the small intestine, are detected by this method. Young et al. (1993) compared NADH diaphorase reactivity with immunoreactivity for a "home raised" antiserum derived from human tumour cells which immunostained cell bodies of myenteric neurons. Their results revealed that the NADH diaphorase method left approximately 20% of neurons in the guinea pig ileum unstained. Johnson et al. (1998) studied neuronal density in the rat small intestine, as determined by NADH diaphorase histochemistry and by immunohistochemistry for a neural marker, protein gene product 9.5 (PGP 9.5). The authors reported that NADH diaphorase histochemistry stained less than 50% of neurons when compared with PGP 9.5 immunohistochemistry and that the number of neurons detected by the former method decreased with age, suggesting that PGP 9.5 immunohistochemistry was the better method for use in aging studies. Johnson et al. (1998) estimated the total number of myenteric neurons to be 19600 ± 2030 per cm² in the 4 month rat ileum, which is substantially greater than Santer's estimate, although neither author reported the extent of stretch during tissue preparation which could potentially affect estimates of neuronal density.

Estimates of total enteric neuronal density vary greatly (as discussed further in results chapters) and factors that may be responsible for such wide variation could include variation in the calculation of intestinal area, such as determination of the original degree of gut distension and/or tissue stretch during preparation. Selectivity of the staining method used by different authors, as well as interstrain and interspecies differences may also be contributing factors.

Although estimates differ considerably, two trends in neuronal packing density have been identified; a greater packing density in the large over the small intestine and on the mesenteric over the non-mesenteric side of the gastrointestinal tract.

1.3.2 Morphological Studies

The first descriptions of the enteric plexuses were made over a century ago by Auerbach (myenteric plexus, 1862) and Meissner (submucous plexus, 1857), see Costa et al. (1987) for review. Many reports of the arrangement of ganglia and neuronal morphology followed, and attempts were made to correlate neuronal morphology and function. Dogiel published important papers on the morphology of enteric neurons (1896 and 1899). The second, more extensive paper described 3 types of neuronal morphology in the myenteric and submucous plexuses of the rabbit, rat, dog and cat, as revealed by methylene blue staining prior to fixation. Dogiel also speculated on the function of the three types of neuron he had identified. He suggested that Type I neurons, which had one long thin and many short irregular processes, were motoneurons. Type II neurons with their many long processes, were thought by Dogiel to be sensory neurons. Type III neurons were described as having processes of intermediate length which branch around the ganglion cells of the same or adjacent ganglia; although Dogiel does not appear to have assigned a specific function to this type of neuron and he only published two drawings to illustrate their shape (Dogiel 1899).

Although Dogiel's classification system is still used today, some workers have disagreed with his conclusions concerning the function of the different neuronal types (Singaram and Sengupta 1996; Song et al. 1991) while others disputed both his classification and conclusions, arguing that although the types of neuron he had described could be identified, many intermediate forms also existed (Brehmer et al. 1999; Furness et al. 1988; Lomax et al. 1999; Stach 1989).

Whereas subsequent studies have revealed neuronal types that correspond to Dogiel types I and II in many species and regions, many intermediate forms exist. Using intracellular injection of Lucifer yellow, Furness et al. (1988) revealed a further two classes of neuron in the guinea pig small intestine. Stach (1989) revealed a further 8 classes of neuron using a silver impregnation technique. More recently, classification of neuronal types has involved more than shape. Attempts have been made to “code” enteric neurons on the basis of their morphology, electrophysiology, neurochemistry and function (see section 1.5). However it is clear from these studies that there are significant differences in the characteristics of enteric neurons both between species and between regions within a single species for example (Clerc et al. 1997; Lomax et al. 1999).

1.4 Ultrastructural studies of the enteric nervous system

Detailed ultrastructural studies of the enteric nervous system have been performed (for example see Gabella 1972; Gabella 1982; Gershon et al. 1981; Kablar 1995; Noda 1986; Rothman et al. 1986), and although there have been relatively few electron microscopic (EM) studies of the GI tract compared with light microscopic studies, there appears to be good agreement between the descriptions of the organisation of enteric ganglia made by different authors on different species.

Characteristics of the adult myenteric plexus have been described in detail in a number of ultrastructural studies of the guinea pig small intestine. Myenteric ganglia are compact structures, isolated from surrounding smooth muscle and connective tissue by a basal lamina (Cook and Burnstock 1976a; Cook and Burnstock 1976b;). The basal lamina has been described by different authors as being an incomplete (Gabella 1979; Gabella 1981) or a complete (Gershon and Bursztajn 1978) sheath around myenteric ganglia.

It has been suggested that the basal lamina may act as a blood-myenteric plexus barrier (Gershon and Bursztajn 1978). In the guinea pig, the segregation of myenteric ganglia by a basal lamina occurs gradually during development and is complete during early postnatal life (Gabella 1982). The presence of a basal lamina surrounding submucous ganglia has not been reported. Myenteric ganglia have been shown to contain neurons, glial cells and a dense synaptic neuropil, with all space within a ganglion being occupied by neuronal and glial elements. A wide variety of neuron and glial cell perikaryon profiles have been reported, as might be expected given the complexity of the enteric nervous system. Ultrastructural descriptions of the submucous plexus are even less numerous than those of the myenteric ganglia, although the features reported are similar to those of the myenteric plexus (Wilson et al. 1981a; Wilson et al. 1981b).

The effects of tissue shrinkage due to fixation and osmication can be substantial (King 1991; Uylings et al. 1986). Such effects however, have not been addressed in ultrastructural studies of the enteric nervous system, especially with respect to the shape and size of enteric ganglia, or the size and shape of individual neuronal and non-neuronal elements.

1.4.1 Enteric Neurons

Myenteric neurons exhibit a great variety of cell soma size and shape, although characteristically they have a large, eccentrically placed nucleus which has few chromatin condensations and prominent nucleoli (Cook and Burnstock 1976a; Gabella 1972). Enteric neurons can be distinguished from glial cells by the size and shape of their nuclei, as well as the electron density of their cytoplasm (Gabella 1981). Attempts have been made to classify myenteric neurons on the basis of the ultrastructural detail of their soma and nerve processes, although no correlation has been found between neuronal subtypes identified at the light microscope level and neuronal profiles identified at the electron

microscope level. Evidence suggests the existence of more different neurochemical types of enteric neuron than have been identified ultrastructurally (Furness and Costa 1980; Llewellyn-Smith et al. 1983), implying that some profiles may correspond to more than one neurochemical phenotype.

Classification of nerve processes has largely focused on transmitter vesicle content, however the morphology of neuronal processes (especially with regard to vesicle content) varies so widely that classifications have been difficult and have been regarded as by some as being tentative (Gabella 1982). Between 3 and 8 types of neuronal process have been identified; (Baumgarten et al. 1970; Cook and Burnstock 1976a; Gabella 1992; Komuro et al. 1982). Such studies have been complicated by the existence of profiles that contain an uneven distribution of two or more vesicle types (Cook and Burnstock 1976b; Gabella 1982). Unlike other autonomic neurons, not all myenteric neurons are covered by a sheath of glial cells, some are in direct contact with the basal lamina, providing the opportunity for direct action of muscle-derived factors on enteric ganglion cells (Gabella 1982).

1.4.2 Enteric Glial Cells

Enteric glial cells were originally referred to as Schwann cells (Gabella 1972; Cook and Burnstock 1976a), although it is now apparent that they are quite distinct from both Schwann cells and satellite cells of the peripheral nervous system. In contrast to other peripheral glial cells, individual enteric glia are not surrounded by basal lamina and their cytoplasm contains 9 – 11µm intermediate filaments and glycogen granules. Some enteric glial cells have been found to resemble central nervous system (CNS) astrocytes (Jessen and Mirsky 1983; Jessen et al. 1983) and contain gliofilaments which are immunologically identical to those found in CNS astrocytes (Bignami et al. 1972; Jessen and Mirsky 1980).

Enteric glial cells appear to be unique among peripheral glia and this unique nature may be important in conferring structural support to myenteric ganglia, especially during mechanical stresses, but at the same time allowing structural rearrangement. In the myenteric plexus of the guinea pig small intestine, glial cells outnumber neurons approximately 2:1 (Gabella 1982), whereas in the submucous plexus the ratio appears to be much closer to 1:1 (Wilson et al. 1981a; Wilson et al. 1981b).

Glial cell profiles can be distinguished by their elongated nuclei, which contain condensed chromatin in abundance, particularly attached to the nuclear envelope (Gabella 1972; Gabella 1981; Gabella and Trigg 1984; Komuro et al. 1982). Glial cell profiles exhibit a wide variety of shapes and sizes, so that systematic classification of these cells has not been possible (Gabella 1982).

1.4.3 Intestinal Smooth Muscle

The ultrastructural features of intestinal smooth muscle have not been well characterised. However, studies of the chicken gizzard and guinea pig *taenia coli* have led Gabella (1992) to suggest that intestinal smooth muscle cells express a homogeneous phenotype and develop simultaneously. Gabella reports that within the same muscle, all muscle cells appeared to be at the same stage of differentiation and he found no evidence to suggest the presence of undifferentiated cells. He suggests that the addition of muscle cells during development and adult hypertrophy is due to division of pre-existing muscle cells, which does not involve de-differentiation. He also found that mitosis was intussceptive i.e. addition of new cells occurred within the muscle rather than at the muscle edges or surface. Chamley-Campbell et al. (1979) suggest that muscle cell density and especially cell-cell contact is important for normal development and maintenance of muscle cell phenotype.

Recent *in vitro* evidence suggests that smooth muscle cells do not respond uniformly to a culture environment, which could perhaps indicate the existence of different phenotypes *in vivo* (Brittingham et al. 1998). As already stated, separation of myenteric ganglia from smooth muscle by a basal lamina occurs gradually during development and is complete during early postnatal life in the guinea pig (Gabella 1982). It is not clear whether this is the case for other species and whether interactions between smooth muscle and myenteric ganglia change as the basal lamina forms.

1.4.4 Interstitial cells of Cajal

Interstitial cells were first described by Cajal (1893) in the rabbit intestine using the Golgi technique and methylene blue staining (see Thuneberg 1982). He described systems of spindle shaped cells with between 2 and 5 primary processes, found between the smooth muscle layers (associated with the myenteric plexus) and at the inner margin of the circular muscle. Methylene blue and zinc/osmic acid impregnation has been used to identify interstitial cells of Cajal (ICC) in two further locations within the intestine wall. Table 1 summarises their locations (see Christensen et al. 1992; Thuneberg 1982).

Table 1 Summary of ICC locations

ICC-I	Located between the longitudinal and smooth muscle layers and associated with the myenteric plexus
ICC-II	Located between the serosa and longitudinal muscle
ICC-III	Located between the circular muscle and the submucosa
ICC-IV	Located between the inner and outer divisions of the circular muscle

The staining characteristics of these cells led Cajal to suggest that they were primitive nerve cells, however subsequent studies have revealed that ICCs exhibit several properties of smooth muscle cells, suggesting a mesenchymal rather than neural origin (see Christensen et al. 1992). These observations raised the issue of whether ICC arise from the neural crest (like enteric neurons and glial cells) or from the mesenchyme (like intestinal smooth muscle).

ICCs in most locations form gap junctions with each other and with smooth muscle cells (see Daniel et al. 1998; Sanders 1992; Thuneberg 1982). ICCs also form close associations with nerve terminals (Wang et al. 1999). ICC also express and respond to a number of neurotransmitters (Grady et al. 1996; Portbury et al. 1996; Shuttleworth et al. 1993; Sternini et al. 1995; Vannucchi et al. 1999; Young et al. 1993).

Electrophysiological studies have revealed that ICCs are involved in the generation of electrical pacemaker activity for GI motility. ICCs generate rhythmic slow waves in membrane potential and control the frequency and the propagation characteristics of gut contractile activity (Ward et al. 1997; Thomsen et al. 1998). ICCs are also thought to act as an intermediary between the smooth muscle cells and enteric neurons (Daniel et al. 1998; Sanders 1992). ICCs can be detected using antibodies raised against stem cell factor receptor (c-kit). This property has been exploited to localise ICCs by immunohistochemistry and to study the role of ICC via the generation of c-kit deficient mice. By using the quail-chick chimera system and probes to label c-kit, ICCs in the avian intestine have been shown to arise from the local mesenchyme (Lecoin et al. 1996). Young et al. (1998) investigated the origin of ICCs in the murine gut by culturing pieces of murine intestine before crest-derived cells colonised the tissue. Immunohistochemistry for c-kit revealed that all classes of ICCs were present, suggesting that in the mouse intestine, as in the avian, ICC arise from the local mesenchyme (Young et al. 1998).

Mutant mice lacking c-kit protein, show intestinal motility disorders and do not exhibit the regular, slow wave contractions observed in the intact animal. Studies of *W/W^v* mice which have a point mutation, reducing but not abolishing c-kit tyrosine kinase activity, also suggest a pacemaker role for these cells. The observation that both excitatory and inhibitory neural inputs to the circular muscle are clearly observed in the intestine of these animals (Ward et al. 1994), suggests that ICCs are not essential intermediaries in this region, however no such inputs were observed in the stomach, suggesting that ICCs may mediate transmission in the stomach.

Although ICCs in different locations have similar morphology and staining characteristics, they do not form a homogeneous population and different ICC subpopulations have been reported on the basis of their differential expression of smooth muscle contractile proteins (Torihashi et al. 1994) and expression of neurotransmitters and their receptors (Grady et al. 1996; Portbury et al. 1996; Shuttleworth et al. 1993; Sternini et al. 1995; Vannucchi et al. 1999; Young et al. 1993). The presence of different subpopulations may indicate functional classes of ICC and that all ICCs may not necessarily have the same embryological origin.

1.5 Neurochemistry of the enteric nervous system

From studies of enteric neuronal morphology and electrophysiology it became clear that enteric neurons were a heterogeneous population, however general staining techniques (such as those discussed previously) do not discriminate between the different subpopulations of enteric neurons. More recently techniques such as enzyme histochemistry, immunohistochemistry and autoradiography have been employed in the identification of enteric subpopulations.

Historically, neural control of smooth muscle activity was thought to be the result of the opposing actions of noradrenaline (released from extrinsic sympathetic inhibitory nerves) and acetylcholine (released from intrinsic parasympathetic nerves), (see Bornstein and Furness 1992; Bornstein et al. 1994; Costa et al. 1987 for reviews). This view was largely undisputed until the 1960's and 1970's when pharmacological and electrophysiological experiments in the presence of both cholinergic and adrenergic blockers revealed that other transmitters were mediating so called non-adrenergic non-cholinergic (NANC) neurotransmission within the enteric nervous system.

It is widely accepted that a number of criteria must be satisfied before a substance can be classified as a neurotransmitter, as listed in table 2. However more recently the situation has been complicated by the identification of substances that may have low (undetectable) storage levels and/or may accumulate by uptake rather than by synthesis (see Furness et al. 1992). Some molecules act to increase or decrease the response of a neuron to a stimulus without necessarily acting as neurotransmitters themselves. These molecules are called neuromodulators. Many of the neuropeptides (e.g. CCK, NPY, see table 4) can influence the excitability of cell by modulation of ion channels and therefore can be said to act as neuromodulators.

<u>Table 2</u>
Criteria for a substance to be classified as a Neurotransmitter
The potential transmitter substance must be produced within a neuron.
The potential transmitter substance must be found within a neuron.
When a neuron is stimulated (depolarised), a neuron must release the potential transmitter substance.
When the potential transmitter substance is released, it must act on a post-synaptic receptor and exert a biological effect.
After the potential transmitter substance is released, it must be inactivated. Inactivation can be through a reuptake mechanism or by an enzyme that breaks downs the substance.
If the potential transmitter substance is applied to the post-synaptic membrane, it should have the same effect as when it is released from a presynaptic terminal following electrical stimulation.
Effects of nerve stimulation should be blocked by antagonists of the putative transmitter substance.

The demonstration of the presence of a neuroactive substance and/or its synthetic enzymes within a neuron is usually taken as evidence that a substance might act as a neurotransmitter. When this information is taken together with distribution and projection information, speculation about the functions of the substance can also be made.

The presence of several putative neurotransmitters has been demonstrated in the enteric nervous system (see Costa et al. 1996; Costa et al. 1987; Schultzberg et al. 1980). Some of these substances and techniques used to demonstrate their presence are discussed in the following sections.

1.5.1 Techniques used to study the neurochemistry of the enteric nervous system

Enzyme Histochemistry

As previously noted, general histochemical stains (such as toluidine blue and methylene blue) have been used to study enteric neurons but they do not distinguish between different neuronal subpopulations. Some histochemical, particularly enzyme histochemical methods however, have proved useful in the location of certain neurotransmitters in the enteric nervous system. Noradrenaline, dopamine and 5-HT (also known as serotonin) are some of the substances that have been detected in nerves by histochemical methods. When reacted with formaldehyde vapor, these transmitters form fluorescent derivatives that are used to locate neurons containing these transmitters (for example Norberg 1964; Jacobowitz 1965). Enzyme histochemistry has been used to detect the presence of transmitter-related enzymes and this technique has been useful where the transmitter of interest is stored in small amounts, stored for a short time or is generally difficult to detect. One example is nitric oxide. This gaseous transmitter is not stored presynaptically, however its synthetic enzyme nitric oxide synthase (NOS) has NADPH diaphorase activity which can convert soluble tetrazolium salts into an insoluble formazan and can thus be used to identify nitrergic neurons (Hope et al. 1991; Scherer-Singler et al. 1983; Vincent et al. 1986 and see section 5.1). Enteric neurons that use acetylcholine as a neurotransmitter have been identified by histochemistry for its degradation enzyme acetylcholine esterase (AChE; Karnowsky and Roots 1964; Koelle and Friedenwald 1949), however the specificity of this reaction has been found to be unreliable.

Autoradiography

This technique has been used to identify neurons that take up specific molecules with high affinity, such as that observed in the reuptake mechanism of neurotransmitter inactivation. This technique has been used at both the light and electron microscope level to study enteric neurons containing γ aminobutyric acid (GABA) (Jessen et al. 1979) and 5-hydroxytryptamine (5-HT) (see Gershon et al. 1992).

Immunohistochemistry

Immunohistochemistry relies on the ability of antibodies to recognise specific molecules (antigens). Antibodies can be detected by a variety of methods, including;

- 1 Direct labelling by fluorescence, colour, enzyme or radioactivity prior to application to the tissue.
- 2 Indirectly, by the application of an unlabelled primary antibody to the tissue and subsequent detection using a labelled secondary antibody from another species that recognises a specific region of the primary antibody.
- 3 The three stage or bridge method which is the most sensitive method. The unlabelled primary antibody is applied to the tissue, then a secondary antibody (raised in a different species) is applied. A tertiary reagent, which may be another antibody, is applied to the tissue and subsequently detected as with the other methods.

The various labels can be viewed directly under ultraviolet or transmitted light microscopy, or in the case of enzyme and radioactive labels, visualised after processing for enzyme histochemistry and autoradiography respectively. The detection of two antigens on the same tissue is possible using a double labelling technique. The tissue is processed for immunohistochemistry with antibodies raised against the first antigen of interest, then the tissue is processed for immunohistochemistry for the second antigen, using antibodies raised in a species different to that used to detect the first antigen.

The immunoreactivity for each antigen is visualised using a different chromophore. Immunohistochemical techniques are generally very sensitive, however a number of factors should be considered when interpreting results obtained with these techniques as listed in table 3.

<u>Table 3</u>	Factors to be considered in the interpretation of immunohistochemical data
Fixation	Fixation conditions are crucial, as different antigens may require different fixation conditions, or even different times in the same fixative. Antigenicity may be lost if inappropriate fixation conditions are used.
Specificity	Antibodies may bind non-specifically to tissue so control experiments where non-immune primary antiserum and omission of the subsequent antibody layers should be carried out.
Cross-reactivity	Primary antibodies may cross react with molecules with similar structure to the immunogen, so absorption or other cross-reactivity controls should be carried out. This is especially important if the immunogen was isolated in a species other than that under investigation. For accuracy, a tissue should be described as having “X”-like immunoreactivity, in case there is cross reactivity with unknown molecules.
Sensitivity	Although immunohistochemical methods are generally very sensitive, the absence of immunoreactivity may not necessarily indicate that the antigen is not present; it may be present in either lower levels than the antibody is able to detect or in a form that is unrecognisable by the antibody, such as a precursor molecule.

With the advent of multiple immunolabelling techniques, it has become possible to investigate the neurochemistry of enteric neurons in more detail. Using a combination of immunohistochemistry and lesion studies, a correlation between neurochemistry, projections and functions for guinea pig enteric neurons has been proposed (Clerc et al. 1997; Costa et al. 1992; Costa et al. 1986; Costa et al. 1987; Gershon et al. 1994; Lomax et al. 1999).

Neurotransmitters present in the enteric nervous system are discussed in the following section.

1.5.2 Neurotransmitters in the enteric nervous system

Noradrenaline

Early pharmacological studies showed that noradrenaline (NA) stimulates smooth muscle relaxation when applied to the intestine. However, subsequent studies have revealed that noradrenergic neurons are not intrinsic to the intestine, but its action on intestinal smooth muscle is indirect via an effect on myenteric neurons (see Costa et al. 1987). Fluorescent histochemical methods and immunohistochemistry for dopamine β hydroxylase (DBH), the enzyme responsible for the conversion of dopamine to noradrenaline, have revealed that in most gut regions and species, sympathetic noradrenergic neurons project to myenteric neurons and blood vessels, as well as the submucous plexus and the submucosa (DBH, Baetge et al. 1990 and fluorescence histochemistry, Jacobowitz 1965; Norberg 1964). Studies in which noradrenergic neurotransmission is disrupted by lesioning the paravascular nerves accompanying mesenteric blood vessels or by treatment with the neurotoxin 6-hydroxydopamine, have revealed that noradrenaline is not the only transmitter to stimulate smooth muscle relaxation (see Costa et al. 1987) and thus started the search to identify the non-adrenergic non-cholinergic (NANC) transmitters.

Noradrenaline may also have a role in the early development of the enteric nervous system, as dopamine β hydroxylase is transiently expressed by a subpopulation of enteric precursor cells (Baetge and Gershon 1989).

Acetylcholine

Acetylcholine (ACh) has been shown to be released by extrinsic pre-ganglionic parasympathetic nerve fibres, as well as to act as an excitatory transmitter in a population of myenteric neurons that supply intestinal smooth muscle and some enteric interneurons (see Furness et al. 1983a; Furness et al. 1984).

Acetylcholine is difficult to detect, therefore enzymes that either synthesise (choline acetyl transferase, ChAT) or breakdown acetylcholine (acetylcholine esterase, AchE) have been used to identify cholinergic neurons. Histochemical methods to detect acetylcholine esterase have allowed descriptions of cholinergic neurons and fibres within the enteric nervous system (Karnowsky and Roots 1964; Koelle and Friedenwald 1949). Subsequently, these methods have proved to be unreliable as non-cholinergic neurons also contain acetylcholine esterase (Koelle 1955). More recent studies have utilised immunohistochemical localisation of choline acetyl transferase to detect cholinergic neurons. Immunohistochemical studies have identified a population of ChAT-containing neurons in both enteric plexuses (Furness et al. 1984; Furness et al. 1983c; Steele et al. 1991; Vannucchi and Faussone-Pellegrini 1996) in several regions of the guinea pig and mouse gut (Furness et al. 1983c). Approximately 69% of myenteric neurons in the guinea pig small intestine were found to have ChAT immunoreactivity (Steele et al. 1991) and approximately 50% of the neurons in the guinea pig submucous plexus have been shown to contain ChAT immunoreactivity (Furness et al. 1984).

Nitric Oxide

It is widely known that a certain class of nerves within the GI wall elicits smooth muscle relaxation. A combination of techniques has revealed that these nerves are intrinsic in origin and are not adrenergic or cholinergic and they are therefore commonly referred to as non-adrenergic non-cholinergic (NANC) nerves.

NANC neurons mediate the majority of inhibitory responses of smooth muscle in the GI tract, including many important physiological reflexes such as relaxation of the lower oesophageal sphincter after swallowing, relaxation of the proximal stomach during eating and descending inhibition in response to distension. The identity of the transmitter used by NANC nerves has remained elusive, however there is good evidence that nitric oxide (NO) is a NANC transmitter (Burt et al. 1990; Toda et al. 1990; Murray et al. 1991; Shuttleworth et al. 1991; Sanders and Ward 1992; Furness et al. 1992; Ivancheva et al. 1998) and that vasoactive intestinal polypeptide (VIP) and adenosine tri-phosphate (ATP) could be two others (Furness et al. 1992; Burnstock 1997;).

Nitric oxide is an unconventional transmitter and is not stored in neurons, like many neurotransmitters but is synthesised on demand in the cytoplasm from L-Arginine by nitric oxide synthase (NOS) and is released from the soma, axon and nerve terminal (see Wiklund et al. 1997; Wiklund et al. 1993). Three forms of nitric oxide synthase (NOS) have been identified; neuronal or brain (nNOS), endothelial (eNOS or Type III) and inducible (iNOS or Type II). The overall homology between these types is approximately 50%. Neuronal NOS has been found to be present in neurons whereas eNOS has been found in endothelial cells and iNOS has been found in macrophages and is inducible in pathological conditions (such as inflammation and inflammatory bowel disease). Once produced, iNOS is continuously active, whereas nNOS and eNOS are inactivated by the binding of calcium-calmodulin allowing local regulated production of NO (see Grisham et al. 1999). To date, 3 isoforms of nNOS have been identified; nNOS α , nNOS β and nNOS γ . Three splice variants of nNOS have been identified in the rat small intestine, however two encode for the same nNOS α protein that has a specific domain for membrane association. The nNOS β protein lacks the domain for membrane association and therefore should be soluble (Huber et al. 1998).

The biological roles of nNOS α and nNOS β proteins are unknown, although they appear to have different subcellular locations, possibly indicating different functions. It is unlikely that either immunohistochemistry or NADPH-diaphorase activity could distinguish between these two isoforms.

Unlike conventional neurotransmitters, nitric oxide is a gas and can readily diffuse through cellular membranes to target surrounding cells. The primary receptor for nitric oxide appears to be soluble guanylate cyclase and its activation results in increased cGMP production and activation of cGMP dependent kinase pathways. It is unclear whether a specialised breakdown mechanism exists to inactivate NO and as it can, in theory, pass through cells, possibly having effects at other sites (see Sanders and Ward 1992). Nitric oxide is however, extremely labile with a half life of seconds in physiological solution, suggesting that a breakdown mechanism may not be necessary.

Neurons which utilize NO as a transmitter have been identified both by immunohistochemistry for nitric oxide synthase (NOS) and by a histochemical reaction where there is a nitric oxide synthase, NADPH diaphorase-dependent conversion of a soluble tetrazolium salt to an insoluble, visible formazan (Scherer-Singler et al. 1983; Vincent et al. 1986). This reaction reportedly only occurs with neuronal NOS, not the endothelial or induced forms (Hope et al. 1991). NADPH diaphorase has been identified as a nitric oxide synthase (Hope et al. 1991) so not surprisingly NOS immunoreactivity and NADPH-diaphorase activity have been shown to co-localise both in culture and in wholemount preparations of myenteric neurons (Belai et al. 1992b; Dawson et al. 1991; Saffrey et al. 1992). There is considerable variation in the estimates of NOS containing neurons in the intestine (see section 5.1) however, fibres of these neurons innervate the circular smooth muscle layer and project anally to other myenteric ganglia and to submucosal ganglia (Furness et al. 1989; Furness et al. 1994).

The possibility of NO being synthesised by glial or smooth muscle cells has not been ruled out as some non-neuronal cells show NADPH diaphorase activity (Saffrey et al. 1994; Schmidt et al. 1992)

Neurons containing NADPH diaphorase have been found to resist amino acid excitotoxicity, hypoxia and degenerative processes associated with ageing, Alzheimer's and Huntingdon's diseases (Dawson et al. 1991; Santer 1994). There has been suggestion that the survival of this population could be due to the catalytic action of NADPH diaphorase and the possible reduction of oxidative neurotoxins (Santer 1994). There may however be changes in nitrergic neurotransmission during ageing as Smits et al. (1996) found that the contribution of nitric oxide to NANC relaxation following electrical stimulation decreased with age in wholemount preparations of rat ileum.

Other transmitters in the enteric nervous system

Apart from the substances discussed in the previous sections, several other substances have been found to have neurotransmitter or neuromodulatory functions in the mammalian enteric nervous system. Table 4 briefly summaries the location and possible roles of some other neurotransmitters in the enteric nervous system. Relevant reviews and original papers are cited. The neurochemical properties of enteric neurons are thus diverse. In addition, many neurotransmitters are colocalised within individual neurons. The existence of several neuronal populations containing different combinations of neurotransmitters shows that the enteric nervous system is more complex than previously thought. It has been suggested that neuronal subpopulations containing particular combinations of molecules may serve particular roles in the gut. Table 5 briefly summarises details of neuronal subpopulations containing combinations of neuroactive substances and possible functions of neuroactive substances in the enteric nervous system (Bornstein and Furness 1992; Costa et al. 1996; Costa et al. 1991; Costa et al. 1986; Gershon et al. 1994; Steele et al. 1991).

Table 4 Putative Neurotransmitters and Neuromodulators in
the enteric nervous system

Adenosine tri-phosphate (ATP)	Interacts with purine receptors (P2) and is involved in NANC inhibitory transmission. ATP is colocalised with nitric oxide in some inhibitory motoneurons. Purinergic receptors have also been found on enteric glial cells.	(Belai and Burnstock 1994) (Burnstock 1997; Grider et al. 1992; Pluja et al. 1999)
Bombesin (BOM)	Stimulates smooth muscle contraction, acetylcholine release, gastric acid secretion and gastric emptying. Release is stimulated by nitric oxide.	(Furness et al. 1988; Schultzberg et al. 1980)
Calcitonin gene related peptide (CGRP)	CGRP immunoreactivity has been detected throughout the intestine and colocalises with other NPY, SOM, CCK and ChAT. CGRP can affect gastric secretion, possibly via actions on gastric bloodflow.	(Clague et al. 1985) (Costa et al. 1986; Costa et al. 1987)
Cholecystokinin (CCK)/Gastrin	Both proteins exist in several forms but share the same COOH terminal pentapeptide. Both CCK and gastrin have been detected throughout the intestine by immunohistochemistry and have been shown to affect acetylcholine release and to stimulate smooth muscle relaxation.	(Furness et al. 1988; Schultzberg et al. 1980) (Schutte et al. 1997)
Enkepalin (ENK)	Immunoreactivity has been found throughout the intestine and in almost all instances to be restricted to the myenteric plexus and nerve fibres in the circular muscle. Suggested roles include inhibition of acetylcholine release and hyperpolarisation of myenteric neurons.	(Furness et al. 1983c)
γ -Aminobutyric acid (GABA)	GABA stimulates both contraction and relaxation of enteric smooth muscle and is thought that the contradictory effects of GABA are mediated by different receptor subtypes, GABA-A receptors mediating relaxation and GABA-B receptors mediating contraction	(Hills et al. 1987) (Costa et al. 1991; Costa et al. 1986; Costa et al. 1987)
Glutamate (GLU)	Recent evidence suggests that this amino acid can stimulate smooth muscle contraction via NMDA receptors in the guinea pig small intestine	(Liu et al. 1997; Sinsky and Donnerer 1998) (Kirchgessner et al. 1997)
5-hydroxy tryptamine (5HT)	5-HT has widespread actions and is found in enteric interneurons, also released on application of luminal pressure to the gut. Suggested roles include stimulation of enteric neurons to initiate peristalsis.	(Costa et al. 1992) (Grider et al. 1992) (Gershon 1999)
Neuropeptide Y (NPY)	NPY immunoreactivity has been found throughout the intestine and is colocalised in adrenergic neurons associated with blood vessels. Possible roles are unknown, however NPY exerts a vasoconstrictive effect.	(Furness et al. 1983b)
Somatostatin (SOM)	SOM immunoreactivity is found in the enteric plexuses and mucosa in several species. It has little effect on smooth muscle cells, although is thought to be utilised by some interneurons. Approx. 5% of myenteric neurons in the guinea pig SI contain SOM immunoreactivity.	(Costa 1980) (Costa and Furness 1984) (Costa et al. 1986)
Tachykinins Substance P, neurokinin-A, -B	Immunoreactivity has been found throughout the gastrointestinal tract of several species. Suggested roles include smooth muscle relaxation, vasodilation, and mucosal functions, as well as stimulating the release of other neuroactive substances. Approx. 14% of myenteric neurons in the guinea pig SI contain Substance P immunoreactivity. Immunoreactivity for NK-1 has also been localised to interstitial cells of Cajal.	(Vannucchi et al. 1997) (Burcher 1989) (Lomax et al. 1999) (Costa et al. 1986)
Vasoactive intestinal polypeptide (VIP) Pituitary adenylate cyclase activating peptide (PACAP)	VIP-ergic projections shown to myenteric ganglia, submucous plexus and coeliac ganglia. Stimulates smooth muscle relaxation, vasodilation and facilitation of mucosal secretion. Approx. 22% of myenteric neurons in the guinea pig SI contain VIP immunoreactivity. PACAP has similar actions to VIP and can act through VIP receptors.	(Costa and Furness 1983; Costa et al. 1992; VanAssche et al. 1999) (Sundler et al. 1992)

Table 5 **Some combinations of neurotransmitters in enteric neurons in the guinea pig small intestine**

Myenteric Plexus	Projections
VIP/DYN/GRP/NADPH-d/NOS	Inhibitory motor projection to the circular muscle
SOM/ChAT/CCK/CGRP/NPY	Secretomotor projections to the mucosa
VIP/DYN	Secretomotor projections to the mucosa
5-HT/ChAT/NFP	Excitatory motor projections to both plexuses
SOM/ChAT +/- Subs P	Interneuron projections to myenteric ganglia
Calret/ChAT	Motorneuron projections to myenteric ganglia and to longitudinal muscle
Calb/Subs P +/- ChAT	Sensory projections to the mucosa and myenteric ganglia
ChAT/NFP/SubsP	Motorneuron projection to circular muscle
ChAT /ENK/CAL	Orally projecting interneuron
ChAT /VIP	Anally directed interneuron
ChAT /SOM	Secretomotor projection
Submucous Plexus	
VIP/DYN/GAL/NMU	NANC secretomotor projections to the mucosa and submucosal arterioles
ChAT/Subs P/CGRP	Sensory projections to the mucosa and myenteric ganglia
ChAT/Calret	Interneuron projections to submucosal ganglia and arterioles
ChAT/CGRP/DYN/CCK/NMU/SOM/GAL	Secretomotor

The above table was compiled using the following references (Bornstein and Furness 1992; Costa et al. 1996; Costa et al. 1991; Costa et al. 1986; Gershon et al. 1994; Steele et al. 1991).

Abbreviations

Calret	Calretinin	NADPHd	Nicotinamide adenine dinucleotide phosphate diaphorase
CAL	Calbindin		
CCK	Cholecystokinin		
CGRP	Calcitonin gene related peptide	NFP	Neurofilament protein
ChAT	Choline acetyltransferase	NMU	Neuromedin
ENK	Enkephalin	NOS	Nitric oxide synthase
DYN	Dynorphin	NPY	Neuropeptide Y
GAL	Galanin	SOM	Somatostatin
GRP	Gastrin related peptide	Subs P	Substance P
5-HT	5-hydroxytryptamine	VIP	Vasoactive intestinal peptide
+/-	May or may not be present		

1.6 Development of the enteric nervous system

1.6.1 Background

Enteric neurons and glial cells are derived from the vagal and sacral regions of a transient and pluripotent structure called the neural crest (LeDouarin and Teillet 1973; Gershon et al. 1992; LeDouarin and Dulac 1992). The neural crest is formed when cells detach from the neural ectoderm at the time of closure of the neural tube. Undifferentiated neural crest cells undergo mitosis and migrate along specified pathways through the embryo until they reach their target destination where they cease to divide, differentiate, make appropriate connections and acquire particular phenotypes. As well as enteric neurons and glial cells, the neural crest also gives rise to melanocytes, neurons of autonomic ganglia and most sensory ganglia. The lineage relationship between the neural crest-derived populations remains uncertain, although there is evidence that a population of enteric neurons and sympathetic neurons share a common lineage in the sympathoadrenal (SA) cells. It has been shown that a population of crest-derived cells which differentiate in the foregut transiently express markers (including tyrosine hydroxylase and dopamine β hydroxylase) expressed by mature sympathetic neurons (Carnahan et al. 1991) and are born early in development, and that some of these cells differentiate into serotonergic neurons (Gershon et al. 1992). A combination of immunological ablation of rat precursors *in vitro* and genetic ablation of murine precursors led to the finding that not all crest-derived enteric cells in the gut express SA markers. Those that do not, include neurons that differentiate later during development, such as those that contain CGRP. This evidence suggests that enteric neurons are derived from at least two different cell lineages (Blaugrund et al. 1996).

Development of the enteric nervous system can be divided into a number of different stages, for convenience. Although the stages have been considered separately below, they may not be consecutive and may overlap.

- 1 Migration of neural crest derived cells to and along the gut
- 2 Commitment to a specific cell (either neuronal or glial) phenotype
- 3 Formation of enteric ganglia and appropriate connections with target cells
- 4 Acquisition and maintenance of neurochemical phenotype

1.6.2 Migration of neural crest derivatives to and along the developing intestine

The migration of neural crest derived cells has been studied both *in vivo* and *in vitro*. *In vivo* studies have been primarily concerned with determining the pathways within the embryo that are used by the migrating cells and with the nature of the extracellular matrix molecules both along these routes and the molecules on the surface of migrating cells. *In vitro* studies have mainly focused on the motile properties of the migrating cells and the effects of various culture substratum on their migration (Akitaya and Bronner-Fraser 1992; Perris 1997).

The use of a chick-quail chimera *in vivo* model system has proved especially useful in determining the origin of enteric neurons and has revealed that cessation of migration and subsequent differentiation of crest derived multipotent precursors is determined by micro-environmental signals (see Gershon et al. 1992; Gershon et al. 1993; LeDouarin and Dulac 1992; LeDouarin and Teillet 1973). Crest derivatives from the vagal region of the neural crest migrate along the dorso-ventral axis through the embryo into the developing mesenchyme and colonise the majority of the presumptive gut (LeDouarin and Teillet 1973).

A smaller number of enteric ganglion cells arise from the sacral region of the neural crest and migrate in an oral direction, colonising the gut as far as the umbilicus (Burns and LeDouarin 1998). The truncal neural crest generates a small population of cells that migrate through the embryo and colonise the rostral foregut (Durbec et al. 1996).

Evidence from both *in vivo* and *in vitro* studies suggest that the microenvironment, particularly extracellular matrix molecules along the migratory route, are important in guiding migratory precursors to their target destination where they undergo differentiation (for example Akitaya and Bronner-Fraser 1992; Bronner-Fraser 1993; Perris 1997). A small population of precursors that reach the myenteric plexus do not differentiate but remigrate and colonise the developing pancreas (Kirchgessner et al. 1992). Once crest derived cells have reached their target, they express characteristic cell surface molecules, such as a 110KDa laminin binding protein (LBP110, for example Pomeranz et al. 1991) and NC-1, (Vincent and Thiery 1984) which are not expressed at other stages of development. This characteristic has been exploited to immunoselect postmigratory crest-derived cells so that their subsequent development can be studied *in vitro* (Chalazonitis 1996; Chalazonitis et al. 1998a; Chalazonitis et al. 1994; Chalazonitis et al. 1998b; Chalazonitis et al. 1995; Pomeranz et al. 1993; Pomeranz et al. 1991). Such studies indicate that the interaction between LBP110 and laminin stimulates postmigratory crest derived cells from the gut to differentiate and acquire neural phenotypes (Akitaya and Bronner-Fraser 1992; Pomeranz et al. 1991)

Impairment of the migration of crest derived cells through particular parts of the intestine result in aganglionosis (lack of enteric ganglia). Aganglionosis is observed in the lethal spotted, piebald lethal, dominant megacolon, endothelin B receptor deficient mutant mice strains and in Hirschsprung's disease in humans (Gershon et al. 1993; Kapur et al. 1996; Kapur et al. 1992; Kapur et al. 1993).

Studies of these conditions suggest that defective colonisation of the large intestine is not always due to the intrinsic properties of the crest derived cells but can be due to abnormal environmental signals which influence migration and/or survival of enteric precursors see (Goyal and Hirano 1996; Kapur et al. 1996; Kapur et al. 1992; Kapur et al. 1993). Evidence suggests that inappropriate extracellular matrix signals such as excess laminin may stimulate premature neurogenesis and cessation of migration, resulting in incomplete colonisation of the gut wall and therefore aganglionosis in the terminal intestine.

1.6.3 Commitment to a neural phenotype

Although enteric neural crest derived cells may be partly committed to a specific phenotype, *in vitro* studies of immunoselected post migratory crest derivatives have revealed that these cells are uncommitted to a specific neurochemical phenotype in the murine, rat or chick intestine when they reach the intestine, implying that the final phenotype is determined by signals from the microenvironment (Rothman et al. 1990; Sextier-Saint et al. 1994). One such signal is thought to be the neurotrophic factor neurotrophin 3 (NT-3) which has been found to stimulate uncommitted precursors to differentiate into enteric neurons and glial cells both alone (Chalazonitis et al. 1998a; Chalazonitis et al. 1994; Chalazonitis et al. 1998b; Chalazonitis et al. 1995) and in combination with other neurotrophic molecules such as epidermal growth factor (Chalazonitis et al. 1994). Basic fibroblast growth factor (bFGF) has been shown to stimulate *in vitro* proliferation of enteric precursors, with leukaemia inhibitory factor (LIF) stimulating their subsequent differentiation (Murphy et al. 1994).

Evidence from *in vivo* tritiated thymidine incorporation studies have revealed that enteric precursors withdraw from the cell cycle and differentiate over a protracted period and that undifferentiated precursors are present in the postnatal murine intestine (Pham et al. 1991). Distinct subpopulations of enteric neuronal precursors can be identified on the basis of time of differentiation, lineage and dependence on a transcription factor (the mammalian homologue of *achaete-scute*, MASH-1) (see Blaugrund et al. 1996; Pham et al. 1991).

Enteric glial cells also develop from the original wave of neural crest cells and do not migrate into the intestine with extrinsic fibres, as was originally thought (see Rothman et al. 1986). Few studies have examined the differentiation of enteric glial cells or acquisition of glial markers such as glial fibrillary acidic protein so the timing of commitment to a glial phenotype and subsequent differentiation is unknown.

1.6.4 Formation of enteric ganglia and appropriate connections with target cells

Once immature neurons have reached their final destination, stable and appropriate connections must be established with target cells before the microcircuits can become functional. This involves not only recognising the correct target cell (type specificity) but in many cases a subset of cells defined by their position within the target area (place specificity). The molecules involved in the recognition process have not been identified, although changes in the composition or distribution of extracellular matrix molecules (such as laminin or collagen) may stimulate aggregation of enteric ganglion cells and therefore ganglion formation (Thiery et al. 1982).

At birth, enteric ganglia are structurally complex, although how they reach such a complex state is yet unknown. Initially, enteric ganglia appear as simple groups of cells which exhibit little difference to the surrounding mesenchyme and these small groups begin to form before the differentiation of the smooth muscle layers and appear to develop independently of other cell types in the gut (see Gershon et al. 1981; Rothman and Gershon 1982).

Differentiating enteric neurons extend processes (neurites) in order to contact target cells. Myenteric neurons project to a range of targets including other myenteric neurons, submucosal neurons and smooth muscle cells. It is unknown whether enteric neurites grow down target-derived neurotrophic factor concentration gradients, as has been observed in other regions of the nervous system (for example, Schohzinger et al. 1994; Thrasivoulou and Cowen 1995). Several molecules have been shown to stimulate neurite outgrowth from enteric neurons *in vitro* (see sections 1.7 and 6.1), although (as yet) there is no direct evidence for target derived neurotrophic factors in the enteric nervous system. There is indirect evidence however, to suggest a relationship between smooth muscle layer thickness and myenteric neuronal size and density. While there is evidence to suggest neuronal density regulates smooth muscle layer thickness and submucosal neurochemical phenotype (See et al. 1990), there is also evidence to suggest that it is the smooth muscle layer that determines myenteric neuronal density and size (Gabella 1987; Gabella 1992; Gabella and Trigg 1984). Clearly there appears to be a relationship between smooth muscle layers and myenteric neurons, although the nature of this relationship and possible mechanisms of its regulation have yet to be described.

In other crest-derived neuronal populations, a period of modification follows target innervation for example, (see Barbacid 1995; Davies 1996; Nishi 1994; Oppenheim 1989; Tolovsky 1997). During this time excess or inappropriate connections are removed by the carefully regulated death of neurons from which the projections originate. This process is called naturally occurring or programmed cell death and sees neurons which do not receive "anti-suicide" signals from target cells undergoing a genetically determined suicide program (see Davies 1996; Oppenheim 1989). It is unknown whether enteric neurons undergo a period of naturally occurring cell death, in part due to the complexity of enteric microcircuits and the difficulty in detecting such neurons.

The development of the submucous plexus has not been well documented, although like myenteric ganglia, submucosal ganglia first appear as groups of morphologically undifferentiated cells that are not easily distinguishable from the intestinal mesenchyme (Gershon et al. 1993; Rothman and Gershon 1982). There is also a suggestion that cells from the myenteric plexus (presumably undifferentiated or partly differentiated precursor cells) remigrate to give rise to the submucous plexus (Kapur et al. 1992; Pham et al. 1991). The observation that the submucous plexus develops both functionally and structurally later than the myenteric plexus (Faussone-Pellegrini et al. 1996; Matini et al. 1997; Vannucchi and Faussone-Pellegrini 1996) would be consistent with this idea.

1.6.5 Acquisition and maintenance of neurochemical phenotype

As mentioned previously, enteric precursors differentiate over a protracted period, implying the co-existence of differentiated neural cells and uncommitted precursors (Blaugrund et al. 1996; Pham et al. 1991). It is probable but unknown whether differentiated enteric neurons and glial cells influence the neurochemical phenotypes of undifferentiated precursors.

Fetal enteric plexuses are in place and responsive to both excitatory and inhibitory stimuli (Gershon and Thompson 1973; Ward et al. 1997) but (at least in the rat) some neurochemical differentiation of enteric neurons occurs during the suckling period and evidence suggests that functional differentiation is not complete until weaning (Fausone-Pellegrini et al. 1996; Matini et al. 1997; Vannucchi and Fausone-Pellegrini 1996).

Several molecules have been shown to influence the development of enteric ganglion cells (as discussed in section 1.7 and 6.1), however factors that may influence neurochemical determination remain undescribed. Differentiation may not simply be the result of the action of a single molecule on a cell but may (more likely) involve a cascade of effects due to multiple factors.

It has been proposed that differentiated cells can influence uncommitted precursors, possibly suggesting alternative roles for neurotransmitters in the developing intestine to that in the mature gut (Pham et al. 1991). Examples are nitric oxide, vasoactive intestinal polypeptide and 5-HT. Whereas nitric oxide acts as an inhibitory neurotransmitter in the adult enteric nervous system, during development nitric oxide appears to be involved in the commitment of some enteric precursors to a neuronal phenotype (Peunova and Enikolopov 1995). A similar finding has been reported for vasoactive intestinal polypeptide (VIP) (Gressens et al. 1993) and pituitary adenylyl-cyclase-activating peptide (PACAP) acting through VIP receptors (Waschek 1996) and 5-HT (Branchek and Gershon 1987).

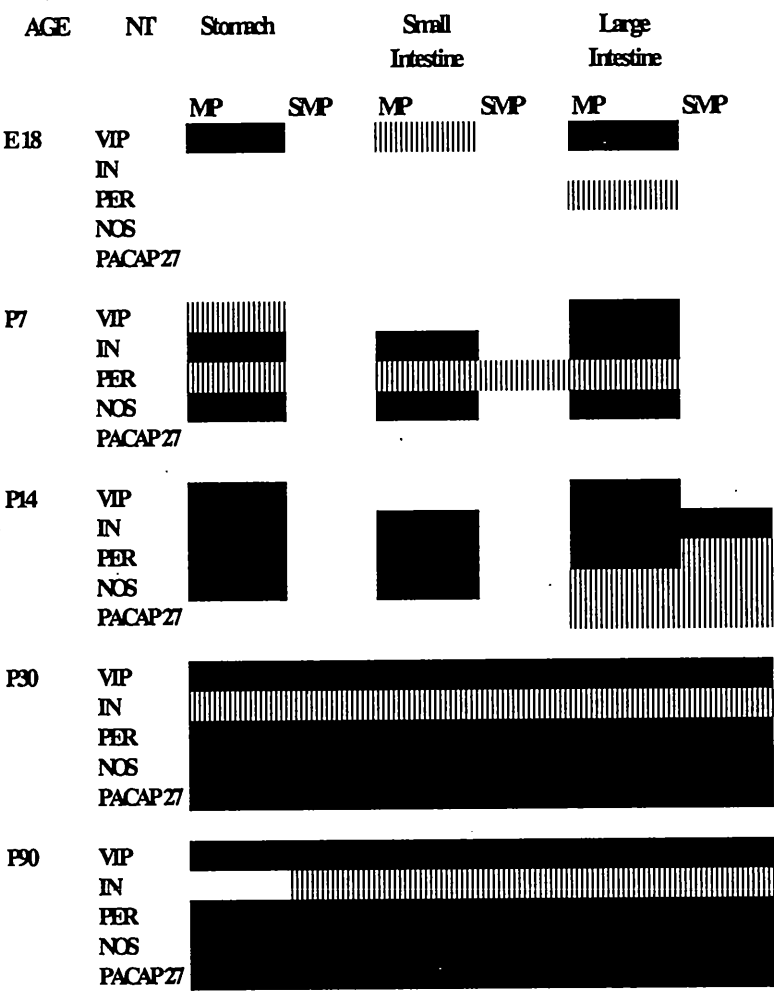
The development of cholinergic neurons, as determined by choline acetyl transferase (ChAT) immunohistochemistry, exhibited a clear, consistent delay between appearance in the myenteric and submucosal plexus (Vannucchi and Fausone-Pellegrini 1996).

Regional differences were also identified in the fetal (18 embryonic days) gastrointestinal tract; the stomach and ileum (but not the colon) containing immunoreactive cholinergic neurons at that stage. The density of cholinergic neurons increased in the colon until the end of the first postnatal week when it appeared to stabilise. This was not the case for the small intestine and the number of cholinergic neurons did not alter significantly between 5 days and 3 months postnatally. Vannucchi et al. (1997) also studied the appearance of Substance P/neurokinin-1 receptors and although these receptors are expressed soon after birth in the rat, it is not until the end of the first postnatal week that the adult pattern of expression is established. The appearance of SP/NK-1 receptors exhibited the same consistent delay between myenteric and submucosal development as described for cholinergic and VIP-ergic enteric neurons (Matini et al. 1997).

The authors also studied the appearance of other transmitters (VIP, PACAP and NOS) from embryonic day 18 (E18) and 90 postnatal days (P90), their findings are summarised in Table 6. Neuronal maturity was also assessed during this time using alpha nexin and peripherin expression. Peripherin is expressed by both developing and terminally differentiated neurons whereas alpha nexin is expressed in high levels by immature neurons but weakly by mature neurons.

In summary, neurochemical phenotype is developmentally acquired during the first month of postnatal life in the rat intestine and phenotypic development of the submucous plexus exhibits a consistent delay behind that of the myenteric plexus.

Table 6 The appearance of some neurotransmitters and marker molecules during development of the rat enteric plexuses (from Matini et al. 1997).



Abbreviations

E denotes age in embryonic days

P denotes age in postnatal days

SMP Submucous plexus

MP Myenteric plexus


VIP Vasoactive intestinal peptide


IN alpha nexin


PER Peripherin

NOS Nitric oxide synthase

PACAP27 an isoform of pituitary adenylyl-cyclase-activating peptide

 Strong staining

 Weak staining

 Staining absent

1.7 Neurotrophic factors and the enteric nervous system

1.7.1 General introduction

During vertebrate development, many neurons depend on their target cells for survival and differentiation. The first identified and best-characterised mediator of such a retrograde trophic action is the neurotrophin nerve growth factor (NGF) (see Levi-Montalcini 1987). Other members of the neurotrophin family have been identified on the basis of structural homology and biochemical properties (see Barbacid 1994; Barbacid 1995; Sariola et al. 1994). The neurotrophin family is comprised of brain-derived neurotrophic factor (BDNF) (Barde et al. 1982), neurotrophin-3 (NT-3) (Enfors et al. 1990; Maisonpierre et al. 1990), and neurotrophin-4/5 (NT-4/5) (Berkemeier et al. 1991; Hallbook et al. 1991). Two additional members, neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) have been identified in fish (Gotz et al. 1994; Nilsson et al. 1998).

Members of the neurotrophin family all bind to a low affinity receptor p75 (p75NTR), however it is the high affinity tyrosine kinase (Trk) receptor that is involved in signal transduction (see Chao and Hempstead 1995). Although there is cross talk between ligands and the Trk receptors, NGF preferentially binds to Trk A, BDNF and NT-4/5 to Trk B and NT-3 to Trk C. Details of the receptors for NT-6 and NT-7 are not yet known.

Observations that NGF increased the survival of developing sympathetic neurons led to the formulation of the neurotrophic theory (Barde 1989; Oppenheim 1989). The neurotrophic theory states that a neurotrophic factor will be present in limiting amounts and rescue a specific population of developing neurons from a period of naturally occurring cell death shortly after neurons have begun to innervate their targets. The neurons that receive the neurotrophic factor survive and those that do not undergo a genetically determined suicide program.

In this manner the abundance of the factor determines the size of the neuronal population, matching innervation to the target size. The assumptions made by this theory are that the putative neurotrophic factor;

- A is present in limiting amounts at the time of naturally occurring cell death
- B supports the survival of a specific population of neurons
- C is target derived

It was soon obvious that the neurotrophic theory could not account for all factors with neurotrophic activity and it has been argued that any molecule which promotes neuronal survival, not just those that conform to the strict criteria of the neurotrophic theory, should be classed as neurotrophic factors (see Barde 1988). This extended definition of neurotrophic activity would also include parameters such as precursor proliferation and neuronal differentiation (i.e. neurite outgrowth or neurochemical phenotype determination), as well as survival. Several molecules have been found to have neurotrophic actions but do not conform to the strict definition of a neurotrophic factor. Examples of such molecules include, acidic and basic fibroblast growth factors (aFGF and bFGF) and ciliary neurotrophic factor (CNTF), which have neurotrophic activity but are not secreted. However, it may not be the free concentration of a putative neurotrophic factor that is important, but the access to it that determines the size of the surviving population (Tang and Landmesser 1993). It has been suggested that some molecules that act as neurotransmitters in the mature nervous system can act as neurotrophic factors in the developing nervous system (Waschek 1996) and see (Cameron et al. 1998). Glial cell mediated trophic effects may also be important in the development and maintenance of neurons and should not be forgotten (see Loughlin et al, 1993).

Another family of neurotrophic factors is the glial cell line derived neurotrophic factor (GDNF) family. GDNF was purified from conditioned medium from cultures of a glial cell line and was found to be a survival factor for dopaminergic neurons and so hailed as a potential therapeutic agent for Parkinson's disease (Lin et al. 1993). Like the neurotrophins, the GDNF family are cysteine knot proteins (Eigenbrot and Gerber 1997) and also like the neurotrophins, they are responsible for the development and maintenance of various sets of sensory and sympathetic neurons. In addition, GDNF family members have been found to play a crucial role in the development and maintenance of enteric neurons and parasympathetic neurons (see Airaksinen et al. 1999). Other members of the GDNF family are; neurturin (NTN, Kotzbauer et al. 1996), persephin (PSP, Milbrandt et al. 1998), and artemin (ART, Baloh et al. 1998). The GDNF family also have roles outside the nervous system - NTN, PSP and ART are expressed in the developing kidney and NTN and PSP have been shown to have *in vitro* effects on kidney development (see Airaksinen et al. 1999), however their *in vivo* roles in kidney morphogenesis are still unclear. Members of the GDNF family mediate signals via a common receptor tyrosine kinase, the Ret (Rearranged during transfection) receptor (Takahashi 1988). A novel class of glycosylphosphatidylinositol (GPI) anchored proteins, the GDNF receptor alpha (GFR alpha) subunits determine ligand specificity; GDNF preferentially binds to GFR alpha 1, NTN to GFR alpha 2, ART to GFR alpha 3 and PSP to GFR alpha 4, in order to activate Ret.

As well as roles in development, there is increasing evidence that neurotrophic factors are likely to be important for the maintenance, survival and repair of the mature nervous system. Such roles have been suggested for NT-3 (see Loughlin and Fallon 1993). It is also unlikely that each neurotrophic factor has just one role within the developing nervous system, as there appears to be considerable overlap of expression and function between different factors.

The overlapping of roles explains in part why the results of “knock-out” mouse studies are difficult to ascertain, as factors with similar actions may take over some of the roles of the lost factor. In culture studies, the effects of combinations of factors or addition of factors in sequence may be different to those when the same factors are applied separately.

1.7.2 Neurotrophic factors in the enteric nervous system

Normal development of the enteric nervous system depends on the supply of several neurotrophic factors at appropriate points in development (see Gershon 1997). The lack of these factors may lead to fatal effects such as those observed in the GDNF deficient mutant mouse (discussed later). As previously discussed (section 1.6), several factors are known to be involved in the embryonic development of the enteric nervous system, however factors that regulate and maintain the postnatal and mature enteric nervous system are less well understood, although the presence of several neurotrophic factors has been detected in the postnatal and adult intestine (see Saffrey and Burnstock 1994). A brief summary of some of the factors detected in the postnatal enteric nervous system and surrounding smooth muscle is given in table 7. *In vitro* systems have been invaluable in assessing the responsiveness of enteric neurons to putative neurotrophic factors, the main features of interest being neuronal survival and neurite outgrowth. Factors that have been shown to stimulate differentiation the *in vitro* development of postnatal myenteric neurons are listed in table 13. To date, there is no evidence that enteric neurons are dependent on neurotrophic support for their survival during a period of naturally occurring cell death, as demonstrated in other regions of the nervous system. This is at least in part because it is technically more difficult to distinguish dying neurons within the widely dispersed ganglia of the enteric nervous system than in sensory and sympathetic ganglia.

Enteric ganglia undergo considerable changes in shape during peristalsis. This is achieved through changes in shape of neurons, glial cells and the neuropil (Gabella 1990). Enteric neurons may require a unique type or level of trophic support in order to survive this constant remodelling, which could be reflected by the action of several neurotrophic factors on the enteric nervous system and the finding that enteric neurons have been shown to be responsive to several neurotrophic factors. Some of the neurotrophic factors and receptors detected in the intestine are summarised in table 7.

Table 7 Neurotrophic factors and receptors present in the mammalian postnatal intestine

Factor	Location	Species	Reference
BDNF	Enteric ganglia	Human	(Hoehner et al. 1996)
GDNF	Intestinal smooth muscle	Rat	(Hellmich et al. 1996; Peters et al. 1998; Silva et al. 1999)
NTN	Smooth muscle, mucosa, submucosa	Rat	(Golden et al. 1999)
NT-3	Enteric neurons	Human	(Hoehner et al. 1996)
Receptor			
GDNFR- α	Muscularis externa	Mouse	(Nosrat et al. 1997)
P75 ^{ntr}	Enteric neurons	Mouse	(Baetge et al. 1990)
Ret	Enteric neurons	Rat	(Golden et al. 1999)
TGF- β	Muscularis externa	Mouse	(Barnard et al. 1993)
Trk C	Enteric neurons	Human Rat	(Esteban et al. 1998; Sternini et al. 1996)

The Neurotrophins

NT-3 has been shown to influence both the embryonic and postnatal enteric nervous system *in vitro* (Chalazonitis et al. 1998a; Chalazonitis et al. 1994; Saffrey et al. 1996; Saffrey et al. 1997). NT-3 promotes the differentiation of enteric crest-derived precursors *in vitro*. Undifferentiated precursors have been found to persist in the postnatal murine enteric nervous system, however it is unknown whether NT-3 stimulates the differentiation of these cells *in vivo*.

Possible roles of NT-3 in the postnatal enteric nervous system are as yet unknown, although NT-3 expression continues into postnatal period and into adulthood and myenteric neurons have been shown to express the high affinity receptor for NT-3 (Trk C) into adult life (Esteban et al. 1998; Sternini et al. 1996). Postnatal myenteric neurons have been shown to respond to NT-3 *in vitro*; showing increased neurite outgrowth and neuronal numbers (Saffrey et al. 1996; Saffrey et al. 1997). Mice that overexpress NT-3 exhibit increased numbers and size of myenteric neurons, indicating that NT-3 may be acting as a survival factor (Pham et al. 1996). Targeted disruption of the neurotrophin (Trk) family of receptors in mutant mice has not revealed specific intestinal abnormalities, such as those reported for GDNF and Ret "knock-out" mice, possibly indicating that the functions of this receptor are taken over by other receptors or that other molecules are able to compensate for the loss.

In the postnatal rat ileum, NGF may have differential effects on the expression of neurotransmitter peptides by enteric neurons (Belai et al. 1992a) and NGF transcripts have been detected in the adult rat gut (see Saffrey and Burnstock 1994).

Ciliary neurotrophic factor

In combination with NT-3, CNTF appears to promote concentration-dependent differentiation of crest-derivatives and postnatal enteric neurons (Chalazonitis et al. 1995; Grider et al. 1997). CNTF may also have a role in the postnatal enteric nervous system as it has been shown to stimulate neurite outgrowth from rat myenteric neurons (Saffrey et al. 1997; and increase the number of myenteric neurons (Saffrey et al. 1997) *in vitro*. CNTF may be involved in the maintenance and repair of the enteric nervous system as CNTF stimulates sprouting of myenteric neurons *in vitro* (Saffrey et al. 1997; Silva et al. 1997). The use of blocking antibodies has shown a dose dependent inhibition of neurite outgrowth, implying that CNTF is endogenous to myenteric ganglia (Grider et al. 1997). The CNTF receptor is tripartite, consisting of an α subunit and two signal transducing β subunits (Davis et al. 1991). The β subunits also act as receptors for other cytokines IL-6 and leukaemia inhibitory factor (LIF) (Stahl and Yancopoulos 1994). The α subunit is expressed in the fetal intestine (Ip et al. 1991; Rothman et al. 1994); mutant mice that lack this subunit die shortly after birth and appear to lack enteric motoneurons (see Gershon 1997).

The GDNF protein family

GDNF and Ret are expressed in the gut during embryogenesis (Pachnis et al. 1993; Watanabe et al. 1997) and mutant mice deficient for either of these proteins lack an enteric nervous system below foregut level (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996). Possible postnatal effects have not been identified in these mutants as they die shortly after birth from renal failure (Schuchardt et al. 1994).

Potential postnatal roles of GDNF in the enteric nervous are unknown, however GDNF and Ret have been detected in the mature enteric nervous system of the rat (Peters et al. 1998), and the mouse (Golden et al. 1999) and postnatal myenteric neurons are responsive to GDNF in culture (Schäfer and Mestres 1999). The presence of another GDNF family member (neurturin) may also have a role in enteric nervous system maintenance, as its presence has been detected in the adult enteric nervous system (Golden et al. 1999) and myenteric abnormalities have been shown in neurturin deficient mice (Heuckeroth et al. 1999). Myenteric neurons of neurturin deficient mice are smaller and appear to extend fewer neurites. Release of peptide neurotransmitters from myenteric neurons and smooth muscle contraction is also decreased in these animals (Heuckeroth et al. 1999). Possible physiological effects of persephin and artemin in the gut are as yet unknown.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

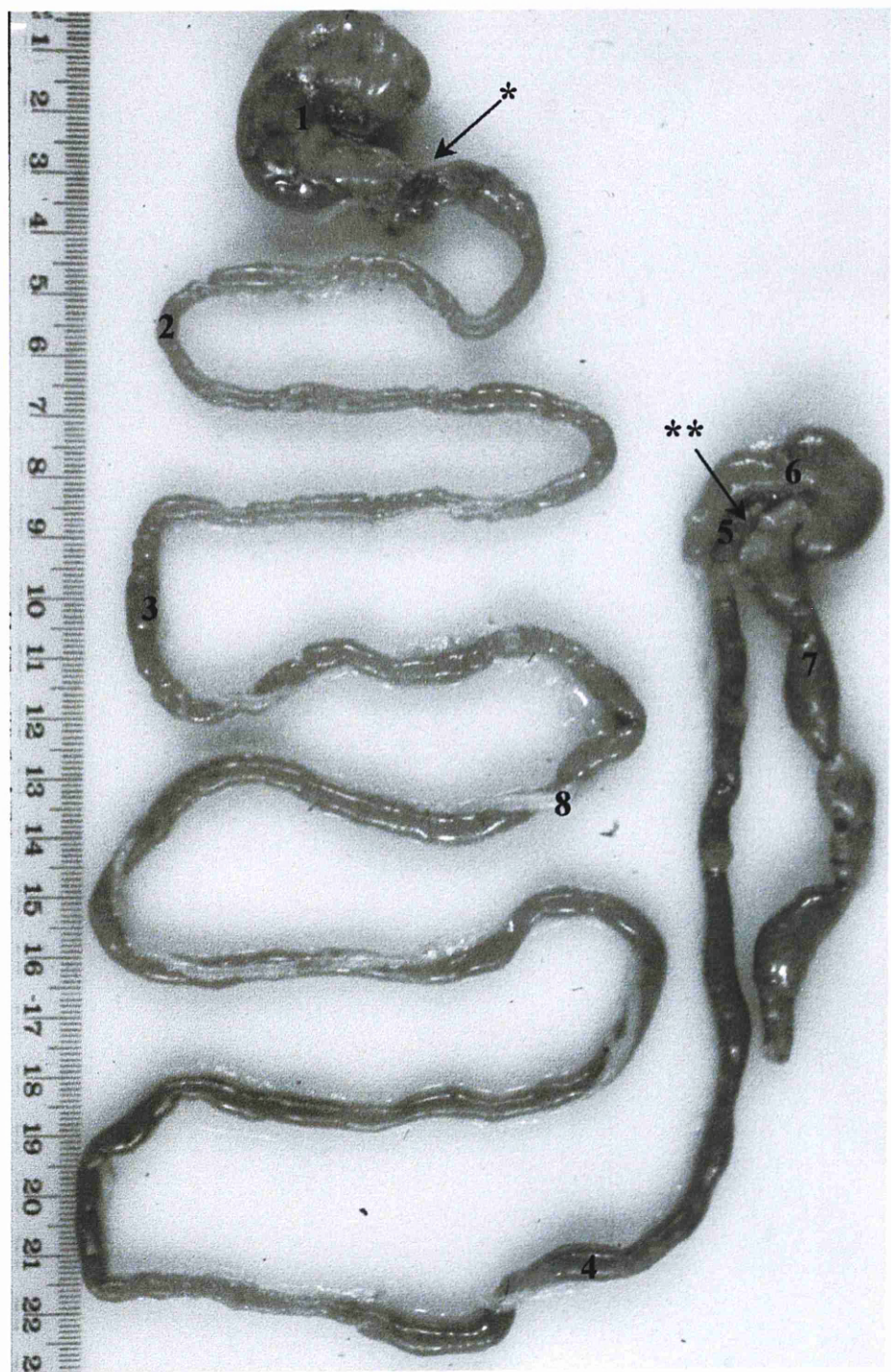
Male rats of the Wistar-derived CHF B strain were used at the following stages of development; postnatal days 7 and 14 (suckling); 21 days (weaning); 28 and 35 days (young adult) and 2 and 6 months (adult). Similar sized litters of animals were used (approximately 10) and the animals were housed with their mother for 21 days after which they were kept at a maximum of four to a cage. They received standard dry food (SDS Universal Ltd., UK, diet 3) and water *ad libitum* and were maintained on a 14-hours light -10-hours dark cycle until sacrifice. Rats were sacrificed using Schedule 1 methods.

2.2 Growth Assessment

General body mass was used as a measure of whole animal growth. Body mass was recorded in grams for each rat and the mean calculated for each age group. Small intestine length and circumference data was used to calculate the mean intestinal area for each age group. This was used as a measure of small intestine growth. Small intestine circumference was measured on tissue that had been opened along the mesenteric border. Measurements were made at five random places along the small intestine and the mean calculated for each animal. An adult small intestine is shown in Figure 2a. Measurements of small intestine length were recorded in centimetres from the gastro-duodenal junction to the ileo-caecal junction (as indicated by the marked arrows in figure 2a).

Figure 2a An adult rat small intestine

Each small intestine was measured prior to fixation. Small intestine length was measured between the junction with the stomach (*) and the junction with the large intestine (**). A labelled adult small intestine is presented below.



1	Stomach	5	Ileo-caecal junction
2	Duodenum	6	Caecum
3	Jejunum	7	Colon
4	Ileum	8	Mesentery

2.3 PGP 9.5 Immunohistochemistry

2.3.1 Tissue Preparation

Male rats were used at the following postnatal days; 7 (n=9) and 14 (n=8); 21 days (n=9); 28 (n=5) and 35 days (n=6) and 2 (n=4) and 6 months (n=6). Following death, the small intestine was rapidly removed, placed into cold (stored at 4°C) Dulbecco's phosphate buffer (dPBS) and its length and circumference measured. The composition of dPBS is given in appendix 1.

The ileum was then removed from the rest of the small intestine, cleaned of mesentery and luminal contents and washed for 2 minutes with an acetic acid rinse (distilled water containing 5% acetic acid and 0.9% sodium chloride; Zhou et al. 1994). Pieces of the terminal ileum were removed, washed dPBS and then cut open along the mesenteric border. The tissue was slightly stretched and pinned onto Sylgard then remeasured to determine amount of stretch. The tissue was then fixed with either Zamboni's fixative (dPBS containing 4% paraformaldehyde and 15% saturated picric acid) or with dPBS containing 4% paraformaldehyde. A range of fixation times was investigated (1 - 18 hours) at both 4 °C and at room temperature. Three hours at room temperature gave good fixation with both fixatives, as determined by quality of PGP 9.5 immunoreactivity. Fixative was removed by three 10 minute washes with 80% ethanol. Tissue was then permeabilised by incubation in dPBS containing 0.5% Triton X100 for one hour at room temperature. The myenteric plexus was then exposed for immunohistochemistry. The muscularis externa was peeled from the ileum then the circular muscle was removed from the muscularis externa and discarded, leaving the longitudinal muscle and attached myenteric plexus.

To standardise the tissue as much as possible between animals, the terminal portion of the ileum was used in each case and the tissue flushed through with dPBS prior to fixation. The amount of stretch during tissue preparation was also calculated so that it could be considered in the area calculations.

2.3.2 Immunohistochemistry

Immunoreactivity for the neural marker, protein gene product 9.5 (PGP 9.5) was used to visualise myenteric neurons. PGP 9.5 is a neuron-specific protein (Thompson et al. 1983) and evidence from previous studies indicate that PGP 9.5 antisera recognise the great majority of enteric neurons (Krammer et al. 1993; Eaker et al. 1994; Karaosmanoglu et al. 1996 and Johnson et al. 1998). Rabbit antiserum raised against human PGP 9.5 (Ultraclone) was used to visualise myenteric neurons using either a peroxidase or a fluorescence method.

2.3.3 Visualisation of PGP 9.5 Immunoreactivity Using a Peroxidase Method

PGP 9.5 immunoreactivity was visualised using the Vectastain Elite Avidin Biotinylated Complex (ABC) peroxidase kit. Endogenous peroxidase was quenched by incubation with methanol containing 0.3% hydrogen peroxide (30 minutes at room temperature). Non-specific antibody binding was blocked by a 30 minute incubation in dPBS containing 1.5% normal goat serum (Vectastain). Excess serum was removed from the preparations, which were then incubated in a humid chamber with antiserum to PGP 9.5 (rabbit anti-human, Ultraclone). A range of antibody dilutions (1:100 - 1:10,000) and incubation times (2 hours - 48 hours) were tested and the optimum was found to be an incubation of 18 hours with a dilution of 1:3000 in antibody diluting solution (ABDS); dPBS containing lysine (1mg/ml), BSA (0.01%), sodium azide (0.05%) and Triton X100 (0.1%). After removal of primary antibody, the tissue was washed and incubated for 1 hour with dPBS containing 0.5 % biotinylated goat anti rabbit antibody and 1.5 % NGS (Vectastain Elite ABC kit). After further washes, the tissue was incubated for 1 hour with dPBS containing ABC reagents from the Vectastain Elite ABC kit. After further washes, immunoreactivity was visualised with a solution containing diaminobenzine (DAB) and nickel (Vectastain). Immunopositive cells appeared blue-black and were observed and photographed under a Zeiss Axiophot light microscope. Immunoreactivity was absent following omission of primary antibody, secondary antibody, ABC reagents or DAB solution.

2.3.4 Visualisation of PGP 9.5 Immunoreactivity by Immunofluorescence

Non-specific antibody binding was blocked by incubation with 1.5% bovine serum albumin (BSA) in dPBS. Excess BSA was removed from the preparations, which were then incubated for 18 hours in a humid chamber with antiserum to PGP 9.5 (rabbit anti-human, Ultraclone) at a 1:3000 dilution as described previously. The tissue was then washed dPBS and incubated with biotinylated donkey anti rabbit antibody (Amersham, 1:250 in ABDS) for 1 hour at room temperature. After further washes, the tissue was incubated with streptavidin-fluorescein (Amersham, 1:100 in ABDS) for 1 hour at room temperature, washed and mounted with citifluor anti-fade mountant (City University, UK). Immunopositive cells fluoresced when viewed under ultraviolet light using a Zeiss Axiophot light microscope fitted with a standard FITC filter set. Immunoreactivity was absent when primary antibody, secondary antibody, or streptavidin-fluorescein was omitted.

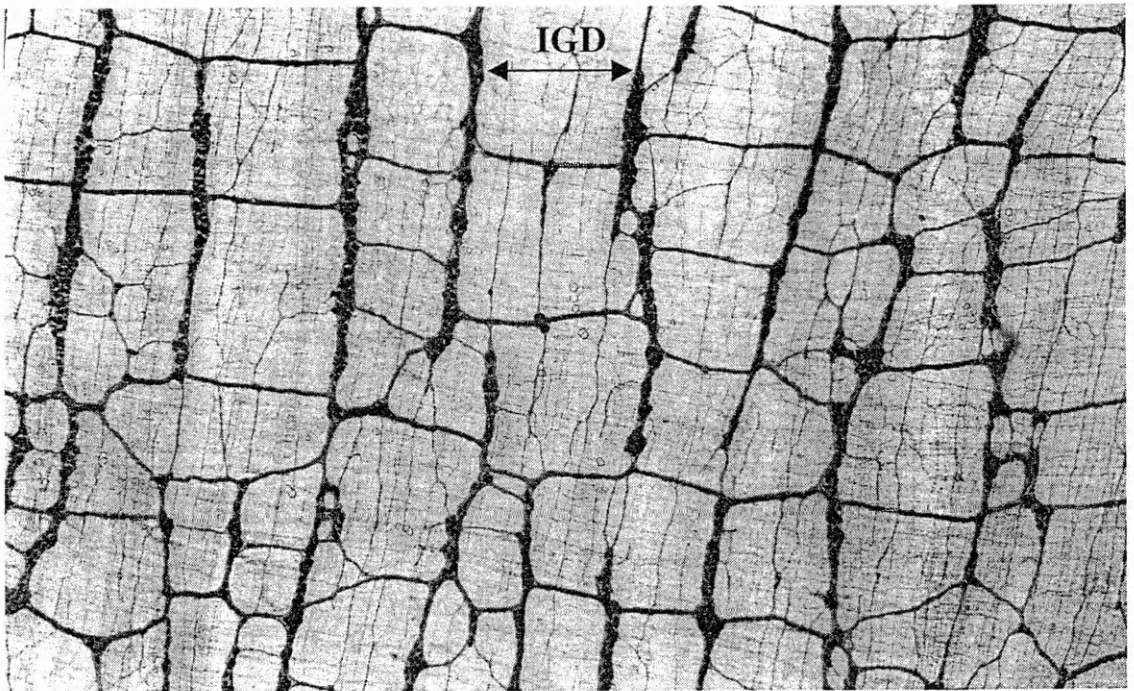
2.3.5 Analysis of Immunolabelled Preparations

Each immunostained tissue was examined under a Zeiss Axiophot and several parameters were recorded on random fields of view of known area. The microscope was fitted with an eyepiece graticule, the area of which was measured under each objective used and was used as a counting frame. Immunopositive cells were counted within the frame as well as those transecting the lower and right edges. Fields of view were taken randomly along the tissue and the total area of tissue examined calculated for each parameter. As the tissue was cut along the mesenteric border, possible differences between the mesenteric and anti-mesenteric regions were not examined.

Interganglionic distance

The distance between individual ganglia was measured using a Zeiss Axiophot connected to a Microcomputer Imaging Device (MCID) image analysis system (Imaging Research Inc., Canada). For each tissue 50 measurements were recorded under a x10 objective. The measurements were corrected for stretch prior to statistical analysis. Figure 2b shows a typical low power image of PGP 9.5 immunoreactive myenteric ganglia and an interganglionic measurement is illustrated.

Figure 2b A measure of interganglionic distance under low magnification



A low power photograph (X5 objective) of a wholemount preparation of 28 day ileum showing PGP 9.5 immunoreactivity, visualised using a peroxidase method. The distance between 2 ganglia, the interganglionic distance (IGD) is indicated by the double headed arrow. Scale bar represents 25 μ m.

Neuronal Numbers

PGP 9.5 immunopositive cells were counted under a x40 objective on a Zeiss Axiophot light microscope. Positive cells were counted in 10 random fields of view, in total an area of 1.52mm^2 for each tissue. The counts were used to calculate neuronal density (neurons per cm^2 of gut) for each age group. In order to make comparisons between rats from different litters and of different ages, neuronal densities were corrected for both the degree of gut stretch during preparation and general gut growth. A growth correction factor was calculated by comparison of small intestinal area of the older ages to the area of the day 7 small intestine (Johnson et al. 1998). The corrected values were then compared to the mean absolute neuronal numbers at the youngest age examined (7 days) to give a ratio.

Neuronal Size

Neuronal area was used as a measure of size and was recorded under a x20 objective of a Zeiss Axiophot connected to an MCID analysis system. The area of 150 neurons was measured in 4 animals (600 in total) at each of the following ages; 7, 14, 21, 28 days and 6 months. Mean neuronal area was calculated and corrected for tissue stretch. Neurons were categorised according to area and the proportion of neurons within each size group calculated.

2.4 Electron Microscopy

2.4.1 Tissue Preparation

Male rats were sacrificed at various stages of postnatal development; 7 days (n=3), 14 days (n=3), 21 days (n=5), 28 days (n=3) and 6 months (n=3). Pieces of proximal ileum (approximately 3 millimetres long) were removed immediately following death, rinsed in 0.1M phosphate buffer and immersion fixed in 0.1M phosphate buffer containing 2.5% glutaraldehyde in for 2 hours at room temperature. The tissue was rinsed in 0.1M phosphate buffer and post-fixed with 1% osmium tetroxide in 0.1M phosphate buffer for 1 hour at room temperature. Following fixation, tissue was dehydrated with acetone and infiltrated with 1:1 acetone:Epon resin overnight. The tissue was then transferred to fresh Epon for 8 hours. Blocks of tissue were embedded in Epon resin in flat moulds and polymerised at 60°C for 24 hours.

2.4.2 Section Preparation

Transverse semi-thin sections (1 μm thick) were cut on a Reichert-Jung OMUE ultra microtome with a glass knife. These sections were stained with Toluidine blue and examined under a Nikon Labophot light microscope to identify appropriate areas for further viewing. Ultra-thin sections (60-80 nanometres) were cut of appropriate areas and collected on pioloform/carbon coated single slot copper grids. Sections were stained with uranyl acetate and lead citrate prior to being viewed and photographed using a Jeol 1010 transmission electron microscope.

2.4.3 Analysis of Electron Micrographs

The ultrastructure of smooth muscle and myenteric ganglia of the muscularis externa was studied at both low and high magnifications. Low magnification (x500) allowed examination of the muscularis externa thickness, whereas higher magnification (x2000+) allowed more detailed observations of individual cell types. A minimum of 30 measurements was made for each age group under each parameter. The general appearance relative to the adult was also assessed, as was the presence or absence of a basal lamina surrounding each myenteric ganglion.

Under low magnification, the total thickness of the muscularis externa, as well as thickness of the individual smooth muscle layers was recorded in microns (μm). The number of muscle cells spanning each smooth muscle layer was also assessed as a measure of layer thickness and requires no correction for shrinkage.

Under higher magnifications, neuronal and glial cells were observed and their maturity relative to those observed in the adult was assessed. Electron micrographs were also used to look for the presence of morphologically immature (possibly undifferentiated cells) and apoptotic bodies within the postnatal myenteric plexus.

2.5 Visualisation of Nitric Oxide Synthesising Neurons using the NADPH diaphorase method

2.5.1 Tissue Preparation

Male rats from at least 3 different litters were used at the following postnatal ages; 7 days (n=6), 14 days (n=6), 21 days (n=6), 28 days (n=6) and 6 months (n=3). Following death, the small intestine was rapidly removed and its length and circumference measured. The ileum was then removed and prepared as described previously in section 2.3. Briefly the tissue was cleaned of mesentery and luminal contents and washed with Dulbecco's phosphate buffer (dPBS). Segments of ileum were cut along the mesenteric border, slightly stretched and pinned on Sylgard. The tissue was then remeasured in order to determine the amount of tissue stretch. A variety of fixation conditions were investigated. Fixation times between 1 and 6 hours were tested with both Zamboni's and 4% paraformaldehyde fixative. Fixation with and without a prior acetic acid rinse (as previously described in section 2.3) was also tested. The NADPH diaphorase reaction product was not detected following fixation with Zamboni's fixative or with paraformaldehyde fixation with an acetic acid rinse. A 90 minute fixation at room temperature with dPBS containing 4% paraformaldehyde gave optimal NADPH diaphorase histochemistry. Fixative was removed by three 10 minute washes with dPBS. The tissue was washed with 80% ethanol prior to incubation in dPBS containing 0.5% Triton X100 for one hour at room temperature. The muscularis externa was peeled from the ileum and the circular muscle removed from the muscularis externa and discarded, leaving the exposed myenteric plexus attached to the longitudinal muscle.

2.5.2 NADPH-diaphorase Histochemistry

Staining was performed as described by Belai et al. 1992. Briefly, the tissues were incubated with 1.2 mM of β -NADPH (Sigma), 0.24 mM nitroblue tetrazolium (Sigma), 15.2 mM L-malic acid (Sigma) and 0.1% Triton X-100 in 0.1 M Tris- HCL (pH 7.6) for between 2 and 3 hours at 37°C. The optimal incubation time was determined by reaction product intensity under a light microscope. It is however, important to note that the optimal fixation conditions for NADPH diaphorase histochemistry were not the same as those for PGP 9.5 immunohistochemistry.

The tissues were then washed with Tris-HCL buffer and either mounted with Citifluor anti-fade mountant or processed for PGP 9.5 immunohistochemistry as described previously in section 2.3. Cells containing NADPH-diaphorase appeared blue and were observed and photographed under a Zeiss Axiophot light microscope. No staining was observed when β -NADPH was absent from the incubation solution.

2.5.3 Analysis

As mentioned in the above section, fixation conditions that gave optimal NADPH diaphorase histochemistry were not optimal for PGP 9.5 immunohistochemistry. The appearance of apparently PGP 9.5 immunonegative myenteric neurons in paraformaldehyde fixed tissue made estimates of neuronal density on double stained tissue potentially inaccurate so adjoining pieces of tissue were fixed with Zamboni's fixative and processed for PGP 9.5 immunohistochemistry. The number of PGP 9.5 immunopositive cells was used to determine neuronal density as described in section 2.3.5. The density of nitric oxide synthesising myenteric neurons was calculated and analysed as previously described for PGP 9.5 immunopositive neurons.

Neuronal Numbers

The number of NADPH-diaphorase positive cells was counted under a x40 objective on a Zeiss Axiophot light microscope. Positive cells were counted in 10 fields of view, a total area of 1.52mm^2 for each tissue. The counts were used to calculate neuronal density (neurons per cm^2 of gut) for each age group, which were then corrected for growth and compared to give a relative density, as previously described in section 2.3.5.

Neuronal Size

Cell body area was used as a measure of size and was recorded under a x20 objective of a Zeiss Axiophot connected to an MCID analysis system. The area of 100 NADPH-diaphorase neurons was measured in 4 animals (400 in total) at each of the following ages; 7, 14, 21, 28 days and 6 months. Mean area was calculated and corrected for tissue stretch.

2.6 *In vitro* effects of neurotrophic factors on myenteric neurons

2.6.1 Tissue Culture

Dissociated rat myenteric plexus was obtained as previously described (Schäfer et al. 1995) and details of solutions required for this method are given in Appendices 2 and 3.

The muscularis externa was removed from the small intestine of 7 day rat pups and rinsed twice in Hank's balanced salt solution (HBSS) containing gentamycin (200µl/ml) and metranidazole (100µl/ml). The tissue was cut into 2-3 mm strips and incubated with collagenase (CLS II, 1mg/ml) supplemented with DNase (10µg/ml) for 30 minutes at 37°C. The tissue was then vortexed for 20 seconds and inspected for freed plexus. At this stage the tissue was partially digested and required a 30 minute further incubation with fresh enzyme. The tissue was then vortexed for a further 20 seconds. Clean pieces of myenteric plexus, that is plexus that was completely separated from smooth muscle, were harvested using a Gilson pipette (20µl). Harvested plexus was stored in HBBS containing 0.5% glucose on ice. Stored plexus was screened and any that was either damaged or incompletely freed from smooth muscle was discarded. Isolated plexus was rinsed twice in HBBS containing glucose and once in calcium and magnesium free balanced salt solution (BBS). Myenteric ganglia were trypsinised in order to obtain a single cell suspension. Trypsin-EDTA 0.5mg/ml, Hepes, (10mM) and DNase (10µg/ml) were added to 1ml of plexus-containing BSS for a 15 minute incubation at 37°C. The tissue was spun at 900 RPM for 2 minutes and the trypsin supernatant removed. Resuspending and centrifuging the tissue with a soybean-derived trypsin inhibitor (5 minutes, 1000 RPM) stopped trypsin activity. After removal of the trypsin inhibitor supernatant, ganglia were resuspended in 1ml defined culture medium (details of which can be found in Appendix 1) containing 10% heat inactivated fetal calf serum (FCS). The ganglia were dissociated to obtain a single cell suspension by aspiration through a 25 gauge syringe needle.

Viable cell numbers were counted in trypan blue and cells were plated in a volume of 150 μ l onto poly-l-lysine coated glass coverslips in 24 well plates at a density of 1×10^4 viable cells per ml (1500 cells per coverslip). After 1 hour at 37°C, wells were topped up with 850 μ l defined culture medium alone or culture medium containing growth factors (see table 8 for final concentrations). Cultures were then returned to the incubator where the cells were cultures for 48 hours and then fixed with dPBS containing 4% paraformaldehyde.

Table 8 Final concentrations of neurotrophic factors added to defined culture medium

Neurotrophic factor	Final concentrations
GDNF Glial cell line derived neurotrophic factor	100pg, 1ng and 10ng/ ml
CNTF Ciliary neurotrophic factor	10ng/ml
NT-3 Neurotrophin 3	10ng/ml

2.6.2 Analysis

The length of processes (neurites) extended by neurons was measured under control conditions and following treatment with GDNF. The total length of neurites was measured for 150 neurons per treatment (50 per coverslip in triplicate). Only neurons that did not contact other cells were considered for this study. Neurite length was measured under a x20 objective on a Zeiss Axiophot microscope and statistical significance was determined using a two way Anova and Honest Tukey post hoc test (Statistica statistics package).

The proportion of neurons and glial cells was calculated for each treatment. The total number of glial cells and neurons were recorded in a strip along the diameter of each coverslip. Under a x40 objective on a Zeiss Axiophot, this corresponded to approximately 15 fields of view and 2.28mm² for each coverslip. Statistical significance was determined using a two way Anova and Honest Tukey post hoc test (Statistica statistics package). Potential effects of trophic factors on myenteric neuronal size were assessed by examining somal area following treatment with GDNF, CNTF or NT-3 and were compared to neurons cultured under control conditions. The concentrations of each factor tested are shown in figure 2c. The somal area of 50 neurons per coverslip was measured under a Zeiss Axiophot x40 objective for each condition. Coverslips were analysed in triplicate for cultures grown under control conditions or in the presence of GDNF, or in duplicate for those grown in the presence of either CNTF or NT-3. Statistical significance was determined using a two way Anova and Honest Tukey post hoc test (Statistica statistics package).

CHAPTER 3

THE DEVELOPING MYENTERIC PLEXUS

3.1 Abstract

The aim of the work presented in this chapter was to investigate possible changes in myenteric neuronal density in the rat ileum during postnatal development. Immunoreactivity for the neural marker PGP 9.5 was used to determine neuronal density in the suckling, weaning and adult rat ileum. PGP 9.5 positive neurons were counted in wholemount stretch preparations and neuronal density was calculated. Following correction for intestinal growth, neuronal numbers in different age groups were compared. The results showed a significant increase in neuronal numbers during the suckling period, followed by a subsequent significant decrease prior to weaning (between 14 and 21 days). From weaning into young adulthood, a significant increase in neuronal numbers was observed. From 35 days until adulthood, the number of myenteric neurons appeared to stabilise to approximately double that observed at 7 days. The possible explanations and implications of the observed changes in neuronal numbers are discussed.

In summary, this study has shown dramatic changes in the numbers of myenteric neurons during postnatal development in the rat ileum, at a time when the rat progresses from liquid to solid food. This study has also shown the adult complement of neurons to be established between 35 days and 2 months of age.

3.2 Introduction

Although embryonic development of the enteric nervous system has been studied in some detail (see section 1.6), less is known about the postnatal development of this system. In some species, such as rats and mice, differentiation of neural precursors and acquisition of neurochemical phenotype has been found to continue into the postnatal period (Matini et al. 1997; Pham et al. 1991; Vannucchi et al. 1997; Vannucchi and Faussone-Pellegrini 1996), suggesting that at birth, when the intestine becomes critical for survival for these animals, the neuronal circuits that regulate intestinal function are incomplete (Faussone-Pellegrini et al. 1996; Matini et al. 1997). The observations that uncommitted precursors persist postnatally in the enteric nervous system and that commitment to a neurochemical phenotype is ongoing at this time, suggest that the number of differentiated enteric neurons within each plexus is incomplete at birth, and that there may therefore effectively be an increase in neuronal numbers postnatally until the adult complement is reached. In the rat enteric nervous system, there is evidence that neurochemical differentiation continues until the third week of postnatal life (Faussone-Pellegrini et al. 1996; Matini et al. 1997), which is about the time when weaning usually occurs (Thompson and Keelan 1986). The aim of this investigation was therefore to make a detailed study of possible changes in neuronal numbers in the myenteric plexus of the rat ileum during the first month of postnatal life.

Previous studies of neuronal numbers in the enteric nervous system have focused on the adult intestine and typically the results have been expressed as neurons per unit area. There are substantial variations in reported estimates, even within individual species. This variation could be due to a number of factors including the method used to visualise the neurons, the method used to determine intestinal area and inter-strain variations.

Examination of possible changes in the enteric nervous system during development, experimental manipulation, disease states and ageing requires reliable staining methods that will allow all enteric neurons to be identified, regardless of their subtype. Such neuron-specific staining methods are also required to assess the proportions of neurons within individual subpopulations. In previous studies, several methods have been employed to assess the total numbers of enteric neurons, as discussed in section 1.3.1. In this study, immunoreactivity for the pan neuronal marker protein gene product 9.5 (PGP 9.5) was used to visualise myenteric neurons. PGP 9.5 is a neuron-specific cytoplasmic protein (Thompson et al. 1983) and antisera raised against the PGP 9.5 protein have been widely used to identify enteric neurons (Eaker and Sallustio 1994; Johnson et al. 1998; Karaosmanoglu et al. 1996; Krammer et al. 1993; Krammer et al. 1994). Protein gene product 9.5 (PGP 9.5) was originally detected by the two dimensional electrophoresis of soluble proteins and is a hydrolase (Wilkinson et al. 1989), with a molecular mass of 27kDa, a protein sequence of 212 amino acids (Thompson et al. 1983). The neuron specificity of PGP 9.5 has led to the suggestion that it may be phylogenetically and functionally related to another neuron specific protein, neuron specific enolase (Jackson et al, 1985). However, there is no evidence to support this suggestion and these proteins are structurally and immunologically distinct (Doran et al. 1983). Immunohistochemical localisation of PGP 9.5 has been widely used to identify enteric neurons (Eaker and Sallustio 1994; Johnson et al. 1998; Karaosmanoglu et al. 1996; Krammer et al. 1993; Krammer et al. 1994).

The method used to determine intestinal area could also lead to variation in the estimation of neuronal density. The extent to which the gut is distended and the tissue stretched during preparation are sources of variation in the measurement of intestinal area and if not standardised between specimens could be sources of inaccuracy.

When comparing total neuronal numbers between animals of different ages, the relative size of the intestine also needs to be considered if samples, rather than the whole intestine are to be used. As the intestine grows, a dilution effect is encountered, in which the same total number in the intestine of different aged animals would appear lead to an apparent decrease in neuronal density per unit area in the older animal, because of the larger size of its intestine. Thus development studies of total neuronal numbers need to take intestinal growth into account.

Postnatal growth of the intestine has not been fully characterised, although there is evidence of biphasic growth in the intestine of the F344/Yit rat strain (Sakata and Setoyama 1997). The authors describe a period of rapid intestinal growth prior to weaning (3rd week of postnatal life), followed by a period of slower growth after weaning until the adult size is achieved. Interestingly, the authors also describe sex differences in the rate of intestinal growth throughout postnatal development, stressing the need for single sex studies. Possible growth related changes in the number of enteric neurons during postnatal development have not been examined previously.

The aim of this investigation was therefore to assess changes in small intestinal growth and myenteric neuronal numbers during postnatal development. Small intestinal length, circumference and area were recorded as measures of intestinal growth. Myenteric neurons were detected using PGP 9.5 immunohistochemistry and the number of myenteric neurons calculated for each group. The materials and methods for this investigation are listed in section 2.3.

3.3 Results

3.3.1 General Growth

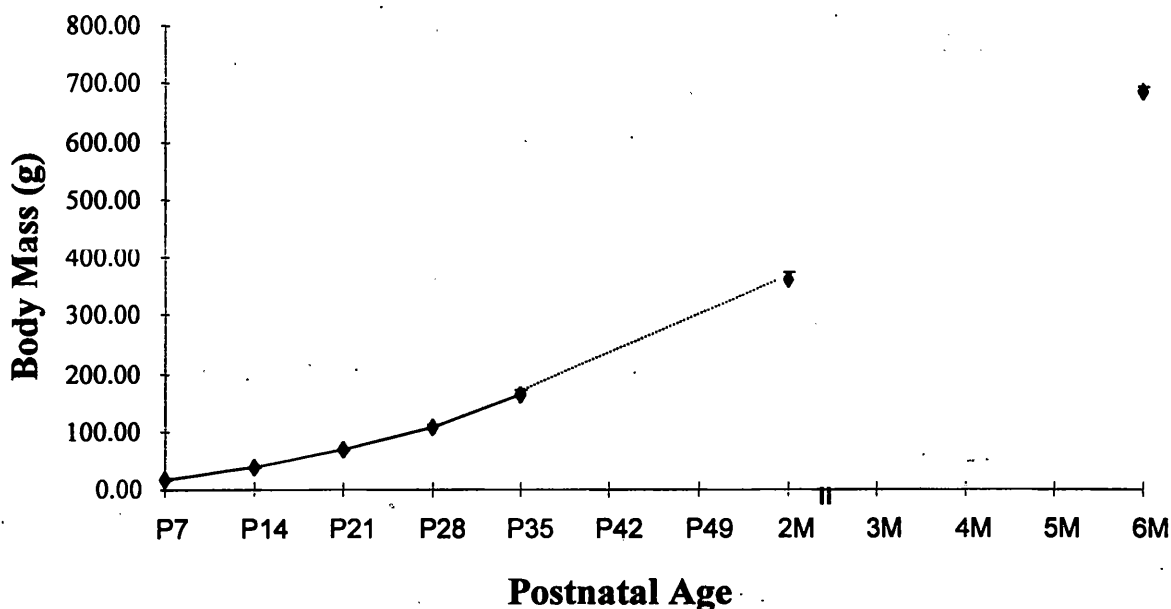
Whole body mass was recorded a measure of postnatal growth in male rats. The rats had received no special dietary or other experimental interventions and were maintained as described previously in section 2.1. The body mass of rat pups increased approximately 40 fold between 7 days and adulthood as shown in figure 3a. The mean mass of a 7 day pup was 17.35g \pm 0.45g. Mass increased by a relatively constant rate of approximately 5 g/day until 35 days, when a mean mass of 161.45 g \pm 9.33 g was reached. Between 35 days and 2 months, whole body growth increased at a rate of approximately 9.5 g/day. Mean body mass increased from 360.30 g \pm 15.80g to 686.14 g \pm 8.69 g between 2 and 6 months. As indicated by the error bars in figure 3a, there was little variation within age groups. The body masses and rates of growth were within values published by Charles River Laboratories, (USA) for similar Wistar strains.

3.3.2 Small Intestine Growth

Small Intestine Circumference

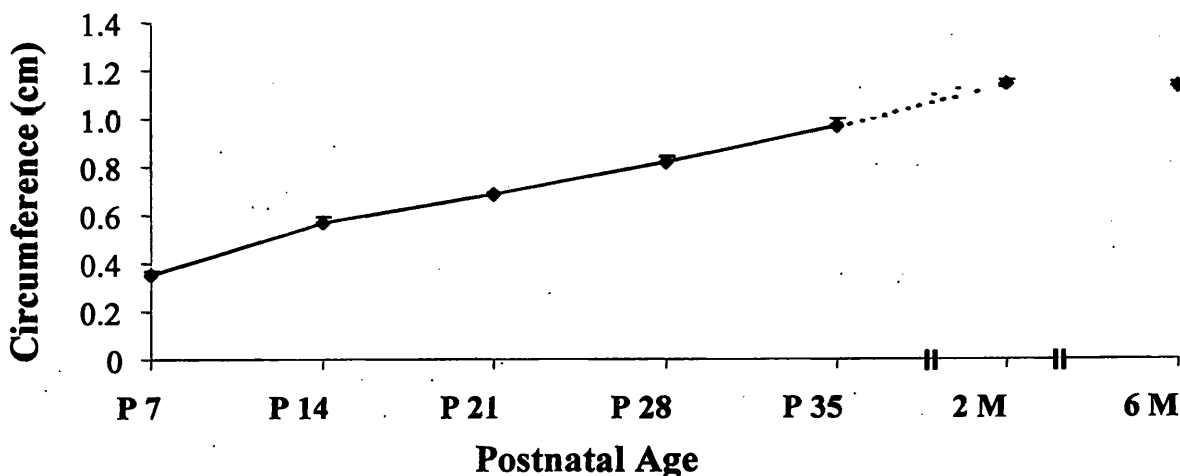
Like body mass measurements, the rate of circumferential growth of the intestine remained relatively constant (at approximately 0.02cm/day) between 7 and 35 days, as illustrated in figure 3b. At 7 days, the mean small intestine circumference was 0.35 cm \pm 0.02 cm. By 14 days mean circumference had increased to 0.57 cm \pm 0.02 cm. The mean circumference at 21 days was approximately double the 7 day mean (0.68 cm \pm 0.01 cm). A further increase was observed between 21 days and 28 days (to a mean of 0.82 cm \pm 0.02 cm), and subsequently between 28 days and 2 months. The mean circumference was (0.97 \pm 0.03) at 35 days and at 2 months the mean circumference was (1.14 cm \pm 0.02). No further increase in mean circumference was observed between 2 and 6 months, the mean intestinal circumference measured 1.13 cm \pm 0.02 cm in the adult.

Figure 3a **Changes in body mass during postnatal development of male rats**



The body mass of rats at several postnatal (P) ages were recorded in grams. The mean \pm standard error are presented. As body mass was not recorded between 35 days and 2 months, a broken line is shown between these points. As the time scale between 2 and 6 months is discontinuous, these points are not joined.

Figure 3b **Changes in mean small intestine circumference during postnatal development**



The circumference was recorded at 5 points along each small intestine and the mean calculated for each animal. The mean for each age group is shown above \pm SE. As measurements were not recorded between 35 days and 2 months, a broken line is shown between these points. As the time scale between 2 and 6 months is discontinuous, these points are not joined.

Animals from at least three litters were used at each age. P denotes postnatal days and M denotes postnatal months.

P7	n=20	P28	n=10	2M	n=10
P14	n=20	P35	n=10	6M	n=10
P21	n=20				

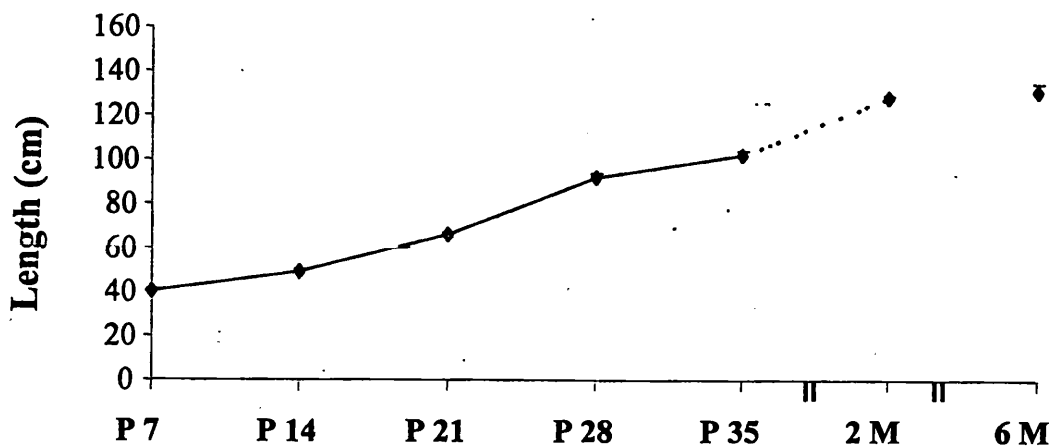
Small Intestine Length

At 7 days, mean intestinal length was 40.50 cm \pm 0.47 cm, which increased to 49.06 cm \pm 1.11 cm by 14 days. Mean intestinal length increased to reach 65.97cm \pm 0.91 cm by 21 days. A further increase was observed between 21 and 28 days (to reach 92.5 \pm 1.59 cm). Between 28 days and 2 months mean intestinal area increased to reach 127.80 cm \pm 1.46 cm. As with circumferential growth, there was no significant increase in length between 2 and 6 months (131.00 cm \pm 4.20 cm), as illustrated by figure 3c. The mean rate of increase in small intestine length was calculated to be 1.22 cm/day between 7 and 14 days. After this initial slow period of growth between 7 and 14 days, the rate of increase in small intestine length rose to a relatively constant 1.87cm/day between 14 days and 2 months.

Small Intestine Area

Mean serosal area was calculated using circumference and length measurements and is shown \pm SE for each age in figure 3d. At 7 days the mean area of the small intestine was 14.13cm² \pm 0.57 cm². Mean small intestinal area almost doubled, to reach 27.28 cm² \pm 0.38 cm², by 14 days. By 21 days, mean small intestinal area had increased further (to 46.00 cm² \pm 1.09 cm²) and to reach 75.47 cm² \pm 1.29 cm² by 28 days. By 35 days mean small intestinal area had reached 98.92 cm² \pm 3.86 cm². There was no further increase in mean small intestinal area between 2 (145.80 cm² \pm 4.54 cm²) and 6 months (148.43 cm² \pm 5.31 cm²).

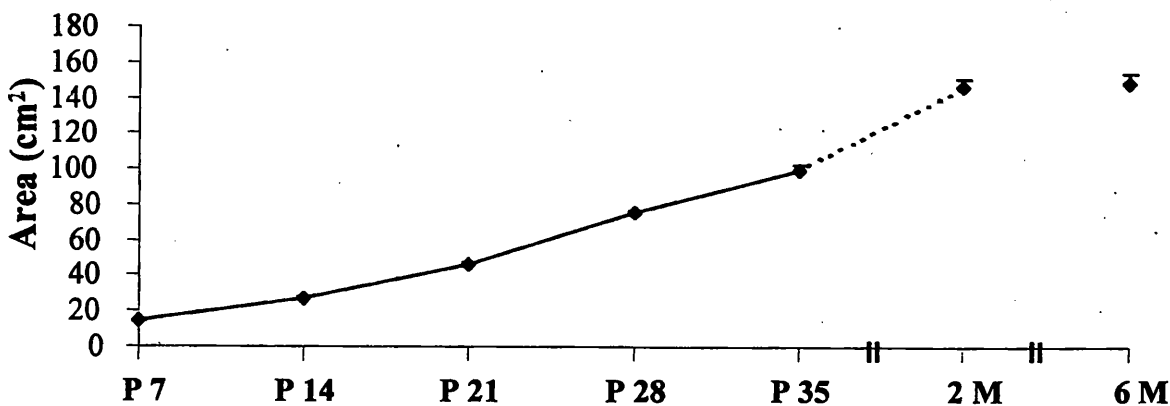
Figure 3c Changes in small intestine length during postnatal development



Postnatal Age

The length of each small intestine was recorded. The mean is shown \pm SE for each age. As measurements were not recorded between 35 days and 2 months, a broken line is shown between these points and as the time scale between 2 and 6 months is discontinuous, these points are not joined.

Figure 3d Changes in small intestinal area during postnatal development



Postnatal Age

The mean small intestine area was calculated using data presented in figures 3b and 3c and is presented \pm SE for each age group. As measurements were not recorded between 35 days and 2 months, a broken line is shown between these points. As the time scale between 2 and 6 months is discontinuous, these points are not joined.

Animals from at least three litters were used at each age. P denotes postnatal days and M denotes postnatal months.

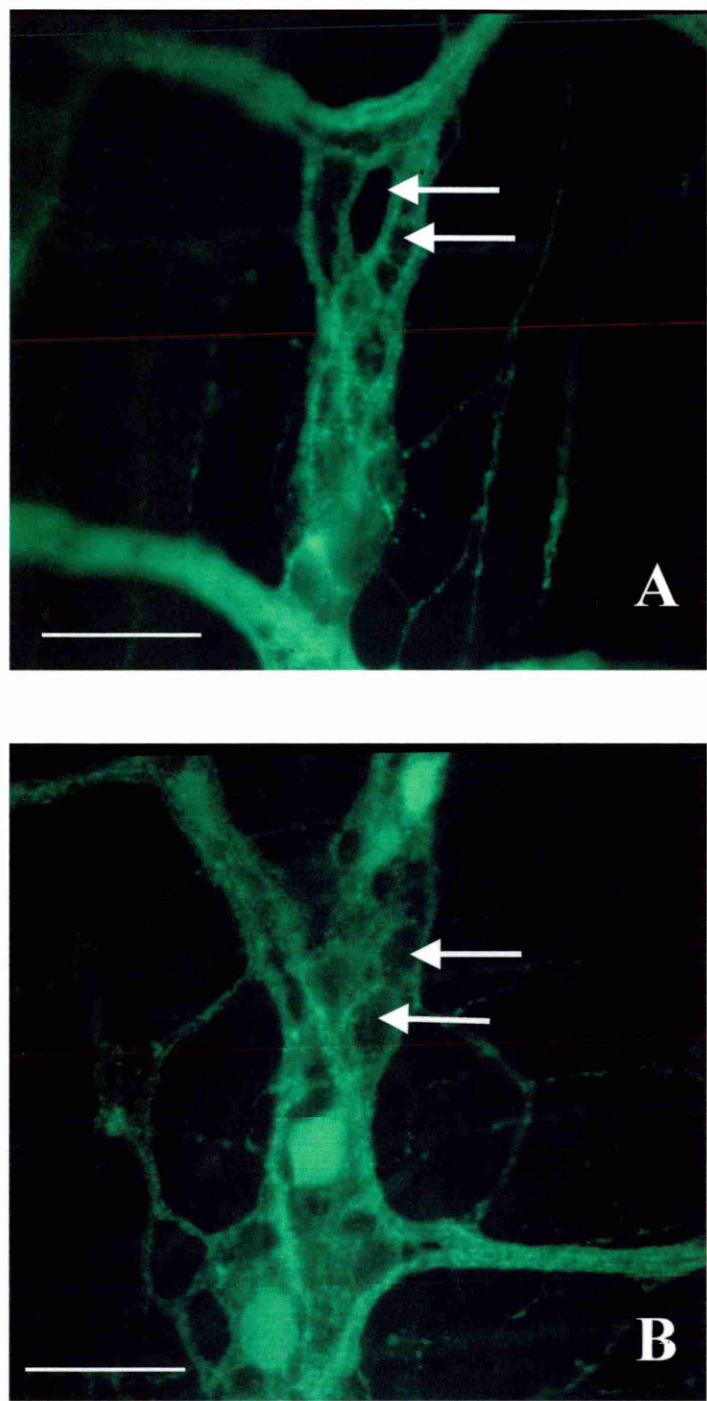
P7	n=20	P28	n=10	2M	n=10
P14	n=20	P35	n=10	6M	n=10
P21	n=20				

Effects of fixation

In wholemount preparations of rat ileum, all myenteric neurons and their fibres appeared to be positively labelled by antisera to PGP 9.5, but were not labelled when the primary antibody was omitted. This does not however, exclude the possibility that a small number of neurons remained unlabelled.

Whilst developing the protocol used for this investigation, interesting observations on the effects of different fixation procedures were noted. When wholemount stretch preparations were rinsed with a dilute acetic acid solution (5% in a sodium chloride buffer) rinse prior to fixation (Zhou et al. 1994) with Zamboni's fixative (4% paraformaldehyde containing 15% saturated picric acid) at room temperature, no cells with a neuronal morphology appeared negative after PGP 9.5 immunohistochemistry. This was not the case however, when either the acetic acid rinse or picric acid were omitted when, although relatively rare, PGP 9.5 negative neurons were apparent and their number increased with age (1% in pre-weaning tissue and approximately 3% in adult preparations). This observation was seen in both peroxidase and fluorescence visualised immunoreactivity. Figure 3e shows PGP 9.5 negative cells in an adult wholemount preparation.

Figure 3e **Effects of fixation: The presence of apparently PGP 9.5 immunonegative myenteric neurons in paraformaldehyde-fixed tissue.**



Photomicrographs showing wholemount preparations of ileum processed for PGP 9.5 immunofluorescence. The tissues were fixed with paraformaldehyde and processed for PGP 9.5 immunohistochemistry (as detailed in section 2.5). The arrows indicate the probable location of cells which are PGP 9.5 immunonegative. These cells were only apparent when treatment with either picric acid or acetic acid were omitted. Image A is a photomicrograph of 21 day ileum and image B is a photomicrograph of 28 day ileum. The scale bar represents 50µm in each case.

General appearance of the myenteric plexus

During development the general appearance of the myenteric plexus changed. At the most immature age, the myenteric plexus appeared as a tightly packed meshwork of ganglia. The ganglia were of irregular shape, so it was often difficult to distinguish boundaries of individual ganglia. The ganglia appeared to be tightly packed with neurons, as shown in figure 3f. As development progressed, myenteric ganglia became further spaced apart and less densely packed with neurons. These observations are illustrated in figures 3f and 3g and reflected in the measurements of interganglionic distance, shown in figure 3h. There was little inter and intra-litter variation with regard to any of the measured parameters, as reflected by the small standard error measurements.

3.3.4 Quantitation of developmental changes

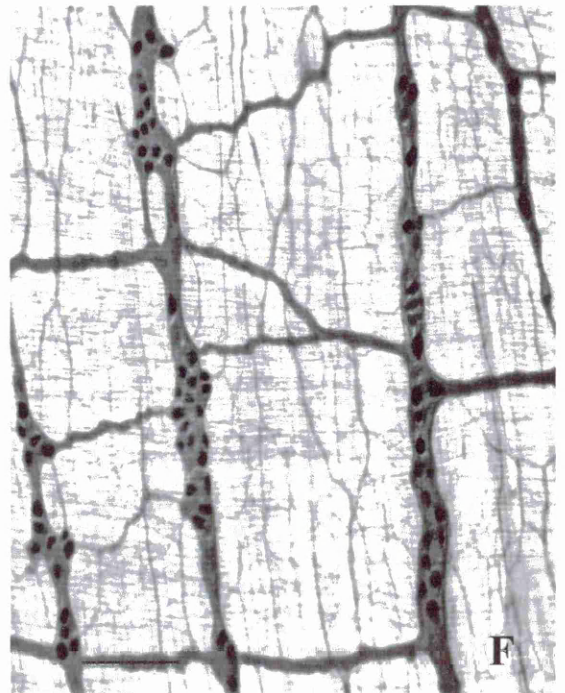
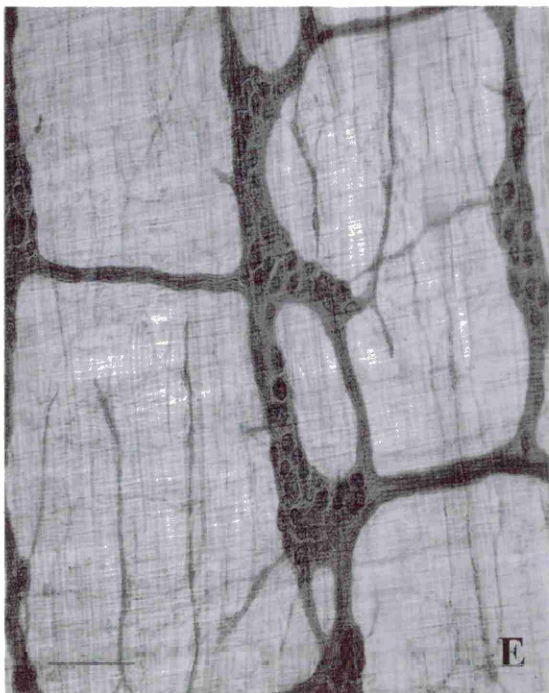
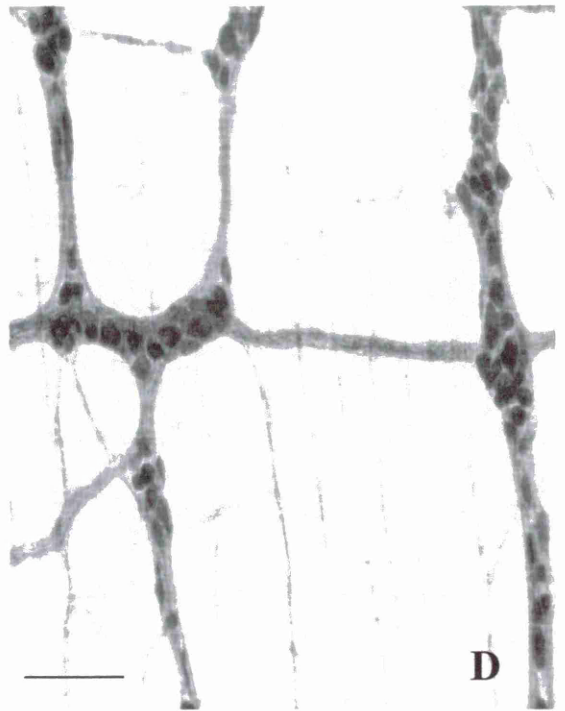
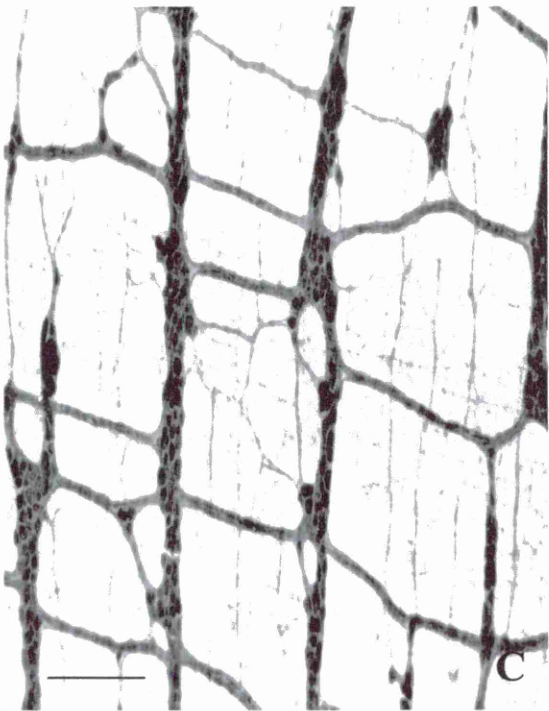
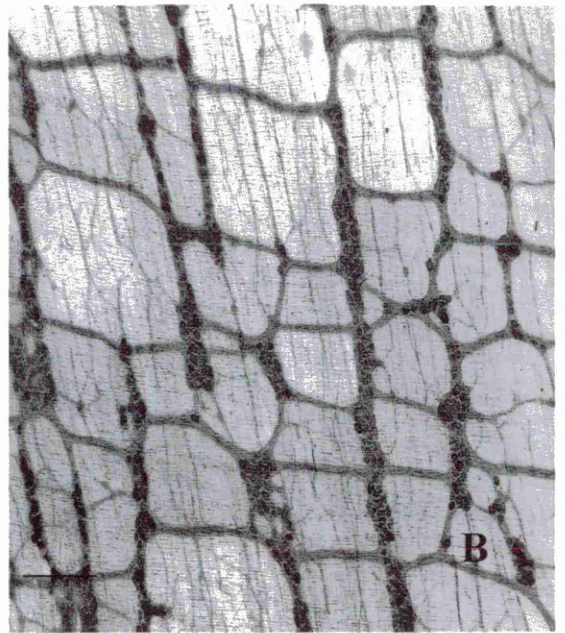
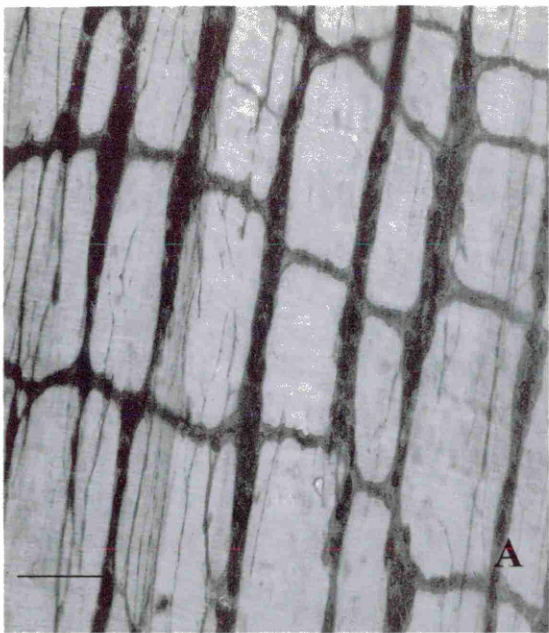
Interganglionic distances

The distance between individual ganglia was measured (as indicated in figure 2b) and the mean of 50 measurements recorded for each tissue. The mean interganglionic distance for each age is presented in figure 3h. At 7 days, the mean distance between individual ganglia was $68.01 \mu\text{m} \pm 1.03 \mu\text{m}$. By 14 days, the mean distance increased almost 2 fold to $123.00 \mu\text{m} \pm 1.44 \mu\text{m}$. From 14 days, the mean interganglionic distance increased until a plateau of approximately $240 \mu\text{m}$ was reached at 35 days.

**Figure 3f Low magnification photomicrographs of the developing
myenteric plexus**

Photomicrographs showing wholemount preparations of rat ileum immunostained for the pan neuronal marker PGP 9.5. Individual ganglia were often difficult to discriminate in young preparations (A), but became increasingly more obvious with age. The scale bar represents 100µm for images A-E and 150µm in image F.

- A 7 day myenteric plexus
- B 14 day myenteric plexus
- C 21 day myenteric plexus
- D 28 day myenteric plexus
- E 35 day myenteric plexus
- F 6 month myenteric plexus



**Figure 3g High magnification photomicrographs of the developing
myenteric plexus**

Photomicrographs showing wholemount preparations of rat ileum stained for PGP 9.5 immunoreactivity. Myenteric neurons appeared blue-black whilst the underlying smooth muscle remained unstained. Immunopositive cells were absent when incubation with the primary antibody was omitted, as illustrated by image H. The scale bar represents 100µm for image A, 50µm for images B-G and 25µm for image H.

- A 7 day myenteric plexus
- B 14 day myenteric plexus
- C 21 day myenteric plexus
- D 28 day myenteric plexus
- E 35 day myenteric plexus
- F 2 month myenteric plexus
- G 6 month myenteric plexus
- H 6 month myenteric plexus processed without primary antibody

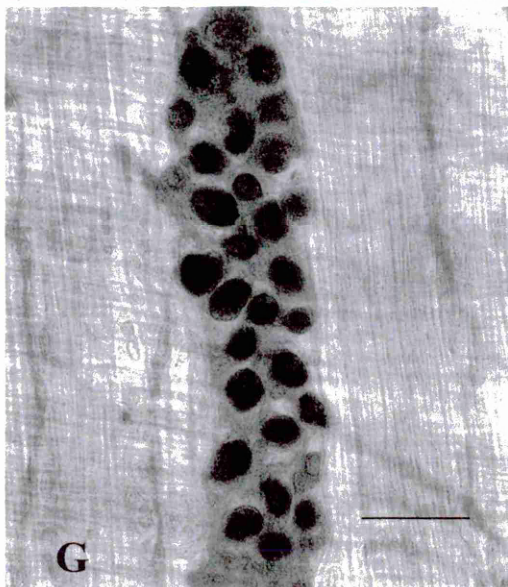
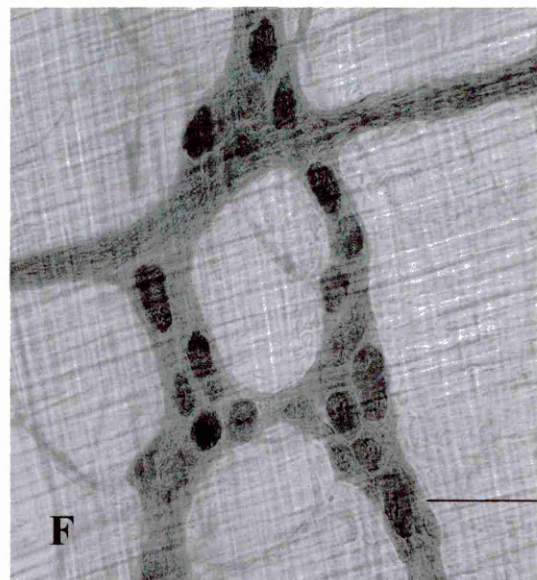
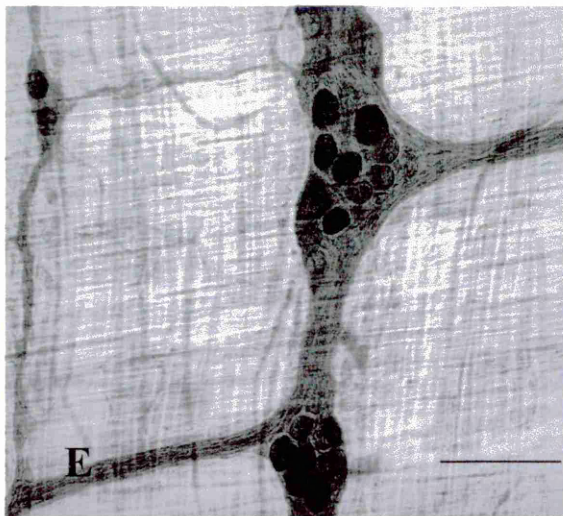
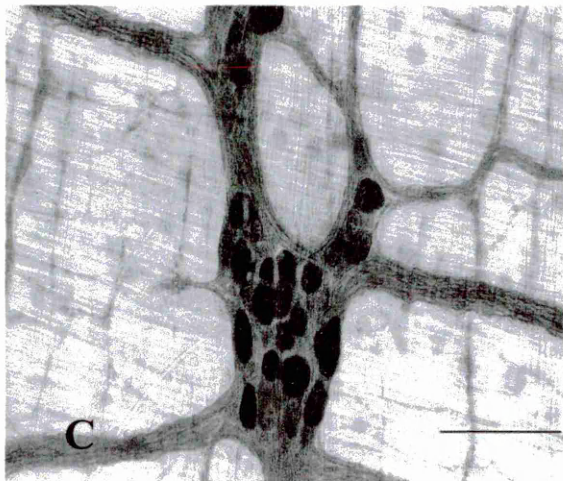
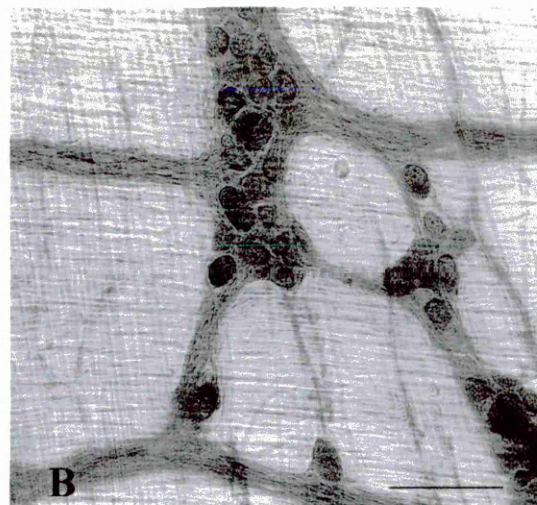
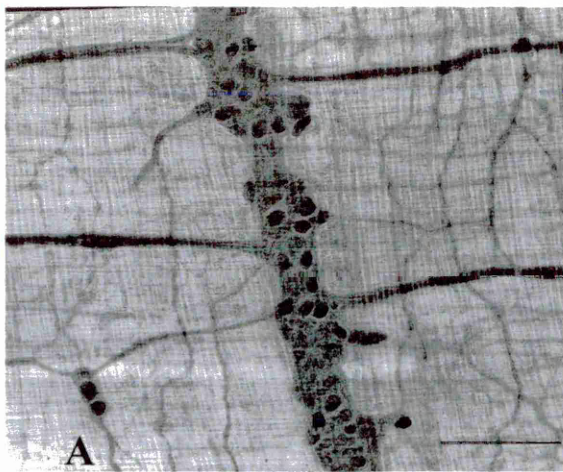
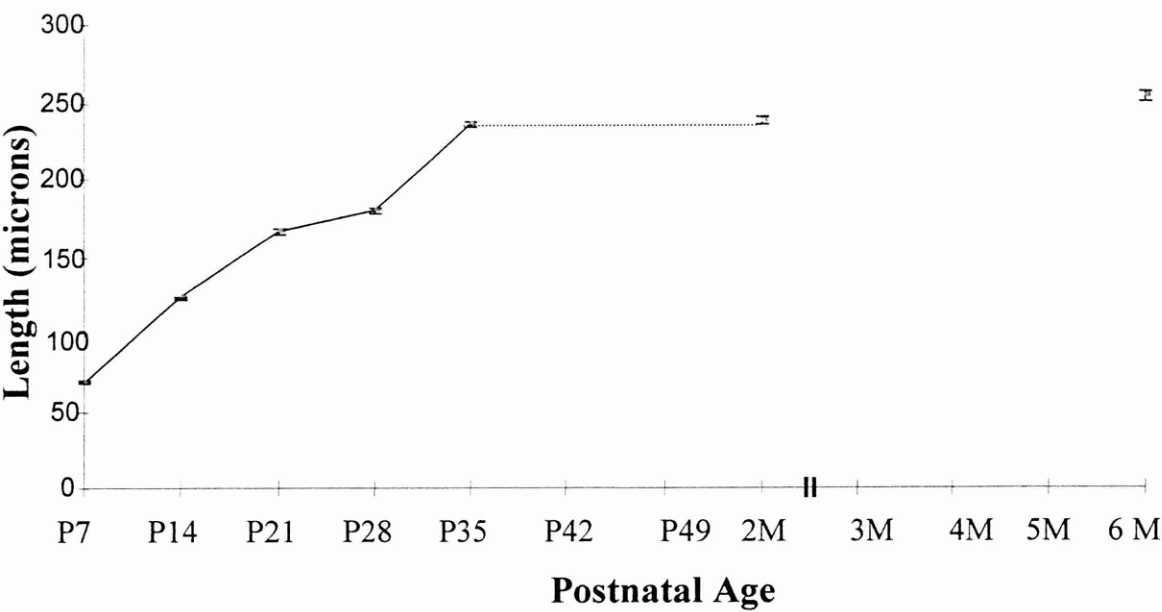


Figure 3h Changes in interganglionic distance during postnatal development



The distance between individual myenteric ganglia was recorded (50 measurements per tissue) and the mean calculated for each age group. The mean +/- standard error is shown for each age group. As measurements were not recorded between 35 days and 2 months a broken line joins the points. As the time scale between 2 and 6 months is discontinuous, these points are not joined.

Animals from at least three litters were used at each age. P denotes postnatal days and M denotes postnatal months.	
P7	n=20
P14	n=20
P21	n=20
P28	n=10
P35	n=10
2M	n=10
6M	n=10

Neuronal numbers

Changes in the total number of myenteric neurons was estimated by counting neurons in a known area of each tissue and expressing the resulting value as density of neurons per unit area. In order to take account of the dilution effect that occurs as a result of small intestine growth, these values of neuronal density were multiplied by a growth correction factor, as discussed in section 2.3.5. The resulting values for each age group are shown in table 9. To aid comparison, after correction for growth, values at different ages were expressed as a ratio relative to the value observed at the youngest age (7 days), as shown in figure 3i.

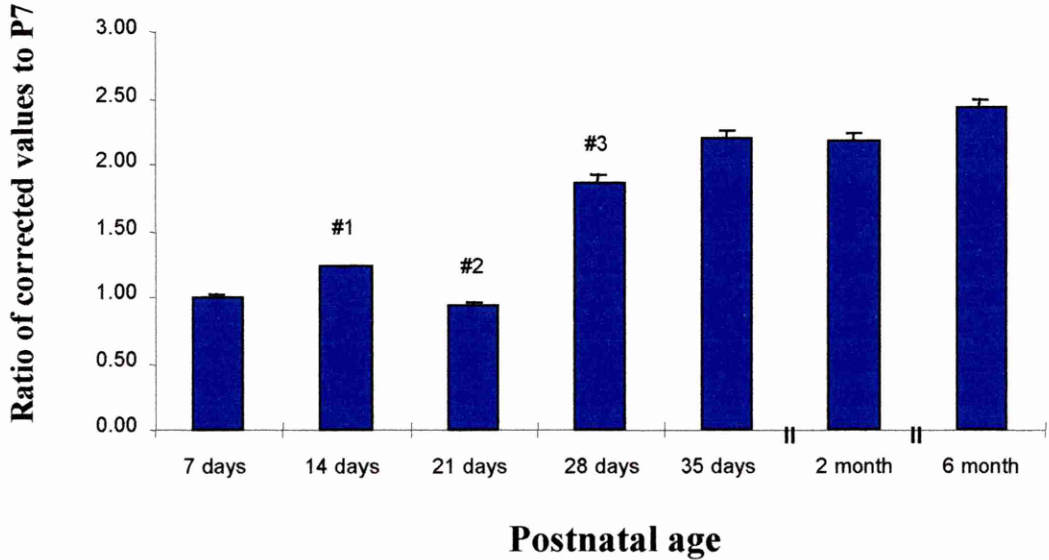
At the youngest age, the mean absolute neuronal density after correction for tissue stretch was 57575 ± 900 neurons per cm^2 of ileum. By 14 days, mean absolute density had decreased to 36714 ± 424 neurons per cm^2 . Following correction for growth, a significant increase in relative neuronal numbers between 7 and 14 days ($P < 0.001$, one way Anova) was revealed, as shown in table 9 and figure 3i. Between 14 and 21 days relative neuronal numbers decreased to a level roughly equal (0.95 ± 0.01) to that observed in the 7 day ileum. This decrease was significant at the $P < 0.001$ level (one way Anova). A large increase in relative neuronal numbers was observed between 21 and 28 days, when the relative neuronal numbers reached approximately 1.87 times that at 7 days. A further increase in relative neuronal numbers was observed between 28 and 35 days to reach 2.19 ± 0.06 . From 35 days onwards, relative neuronal numbers began to stabilise at roughly double that of the 7 day ileum, possibly indicating the formation of a stable pattern of innervation. A slight, but insignificant increase was observed in relative neuronal numbers between 2 and 6 month animals (2.17 ± 0.07 to 2.44 ± 0.06).

Table 9 **Changes in neuronal numbers during postnatal development**

AGE	N	Mean Absolute Density (neurons per cm ²)	Growth Correction factor	Corrected Values	Ratio of corrected values to P7 value
P 7	9	57575 +/- 900	1.00	57575 +/- 900	1.00 +/- 0.02
P 14	8	36714 +/- 424	1.93	70858 +/- 424	1.23 +/- 0.01
P 21	9	16765 +/- 238	3.26	54578 +/- 238	0.95 +/- 0.01
P 28	5	20201 +/- 630	5.34	107873 +/- 630	1.87 +/- 0.06
P 35	6	18050 +/- 392	7.00	126350 +/- 392	2.19 +/- 0.06
2 M	4	12122 +/- 276	10.31	124978 +/- 276	2.17 +/- 0.07
6 M	6	13313 +/- 330	10.50	139787 +/- 330	2.43 +/- 0.06

The chart summarises the mean number of neurons per cm² intestine (absolute density), following correction for general growth (Corrected values; absolute density multiplied by growth correction factor) and in relation to the corrected density at 7 days (corrected values divided by P7 absolute density). The number of animals (N) used at each age is also shown for each group.

Figure 3i **Changes in relative neuronal density during postnatal development**



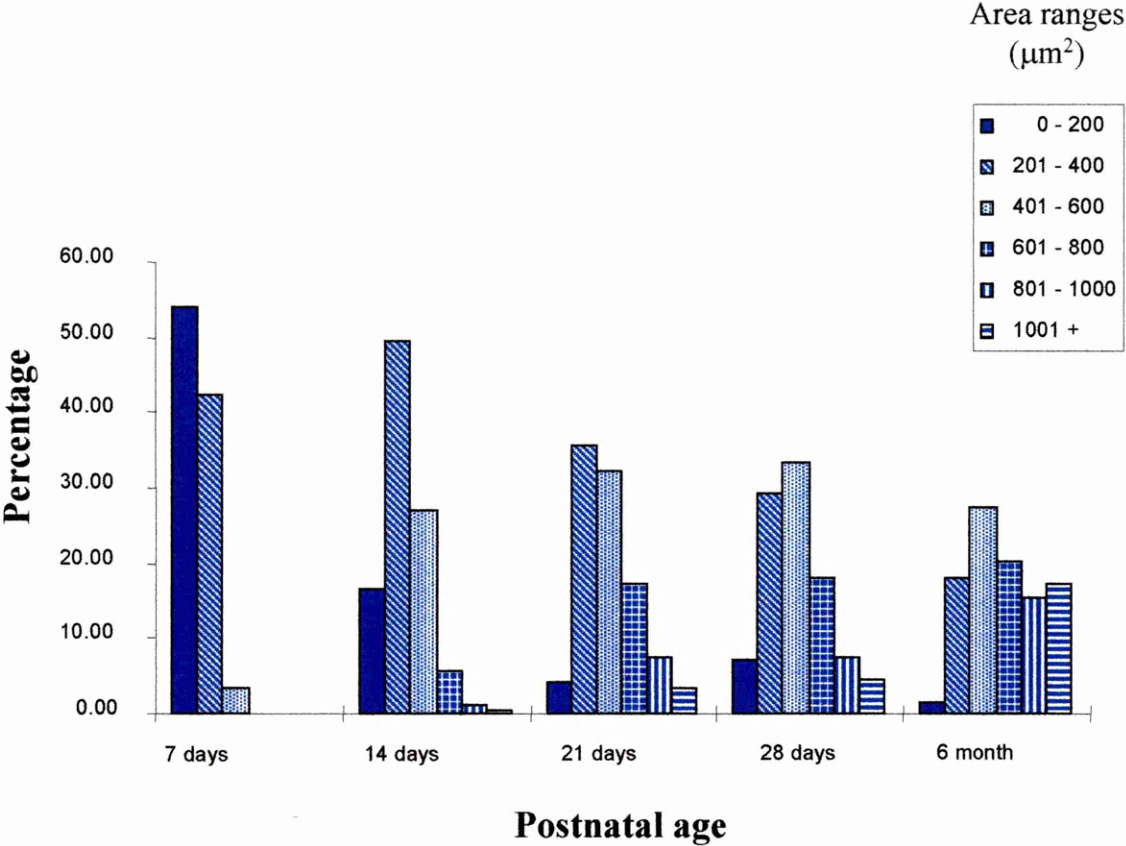
The mean relative values are shown for each group +/- the standard error of the mean. Hatched values (#) are significantly different from the previous value (P<0.001, one way Anova). The mean relative neuronal density by 14 days was significantly greater than that at day 7 (#1), whereas the mean relative neuronal density at 21 days was significantly lower than that at day 14, but not significantly different from that at day 7 (#2). By day 28 the mean relative neuronal density was significantly greater than that observed at day 21 (#3).

Neuronal size

As a measure of neuronal size, the somal area of 600 neurons was recorded for each age group. The mean neuronal area was calculated at each age. Mean neuronal size was significantly larger at 14 days ($353.81 \mu\text{m}^2 \pm 6.59 \mu\text{m}^2$) than at 7 days ($204.44 \mu\text{m}^2 \pm 3.71 \mu\text{m}^2$, $P < 0.001$ one way Anova). There was no significant difference in mean neuronal size between 21 days ($500.15 \mu\text{m}^2 \pm 9.55 \mu\text{m}^2$) and 28 days ($513.16 \mu\text{m}^2 \pm 10.10 \mu\text{m}^2$) although at both ages, neurons were significantly larger than that at 14 days and significantly smaller than at 6 months ($693.52 \mu\text{m}^2 \pm 14.17 \mu\text{m}^2$).

Neurons exhibited a wide range of sizes; variation in neuronal area was observed from the earliest age throughout development, hence to provide a more detailed analysis of growth, the proportions of neurons within size bands of $200 \mu\text{m}^2$ were examined during the postnatal period as shown in figure 3j. The distribution of neuron area exhibited a slight skew so, in order to perform statistical analysis, the data was Log_{10} transformed, which gave a normal distribution. At 7 days, the majority of neurons (54%) were $200 \mu\text{m}^2$ or smaller. In contrast, by 21 days less than 5% of neurons were found in this size range and by 6 months, less than 2% of neurons were within this range. By 14 days, the area of the majority of neurons measured between 201 and $400 \mu\text{m}^2$, which was also the case at 21 days. At both 28 days and 6 months, the area of the majority of neurons measured between 401 - $600 \mu\text{m}^2$. In summary, although neuronal area showed variation throughout postnatal development, in general neuronal area increased with age.

Figure 3j Changes in myenteric neuronal size during development



The area of neuronal cell bodies was categorised into size bands and the proportion of neurons within each size band recorded as percentage of total neurons counted.

3.4 Discussion

This study has evaluated growth of the small intestine and changes in neuronal numbers in the rat ileum during postnatal development. Measurements of small intestine area indicated a substantial (roughly 10 fold) age-related increase between 7 days and 6 months. As intestinal area increases, a dilution effect on neuronal density may occur and this could lead to detection of false decreases in estimates of relative neuronal numbers in animals of different ages. Correction for this effect is essential and was achieved by comparison of neuronal density relative to small intestinal area, as described previously by Johnson et al (1998). After this correction, significant changes in neuronal numbers during postnatal development were detected in the present study.

General and intestinal growth

The rate of whole body growth appeared to be relatively constant during the first five postnatal weeks and was in keeping with published values for rats of similar strains. A constant rate of circumferential growth of the small intestine was also observed. Small intestine length appeared to increase in phases; a relatively slow period of growth was observed between 7 and 14 days, followed by an increased period of growth between 14 days and adulthood. As with circumferential growth, there was no further increase in small intestine length between 2 and 6 months. As the growth of the small intestine occurs at a relatively constant rate through the weaning period, it is unlikely that small intestinal growth changes in response to nutritional changes. This data is in contradiction to observations reported on the small intestinal growth of the F344/Yit rat strain (Sakata and Setoyama 1997). In this strain of rat, a period of rapid intestinal growth was observed prior to weaning followed by a period of slower growth post weaning.

Sex differences in intestinal growth rates were also reported for this strain. Sakata et al (1997) suggest that the rapid growth phase occurs in preparation for dietary change and that when the intestine reaches a level of maturity (as determined by intestinal area), the animal progresses to solid food.

General anatomy of the myenteric plexus

The general appearance of the myenteric plexus (as visualised by PGP 9.5 immunohistochemistry) changed during postnatal development, suggesting that at 7 days the myenteric plexus is immature relative to that of the adult. In the 7 day small intestine, myenteric ganglia appeared to be continuous and densely packed with neurons. As development progressed myenteric ganglia appeared to become more discrete and spread out, however, the boundaries between individual ganglia remained difficult to define, even in older animals. For this reason the number of ganglia per unit area and the number of neurons per ganglia could not reliably be counted, therefore neurons per unit area have been used to estimate neuronal numbers.

Neuronal Numbers

Previous studies have focused on neuronal density in adult animals and there is considerable variation in the neuronal values reported by different authors. This variation could be due to a number of factors including; fixation (see section 3.3), method of neuronal visualisation (see section 1.3.1), the method of area determination (allowing for the relaxation state and stretch during tissue preparation), as well as inter-strain differences. Studies that have utilised NADH histochemistry to quantify total neuronal numbers in the rat, such as those by (Gabella 1987; Santer and Baker 1988), gave estimates substantially lower (roughly 40%) than those presented for the adult rat in this study.

Santer and Baker (1988) estimate 8169 ± 413 neurons/cm² in the myenteric plexus of the 6 month rat ileum, whereas results presented in this study would suggest there to be 13313 ± 330 neurons/cm². More recent reports suggest that the NADH histochemical method does not stain all myenteric neurons as previously assumed and can leave between 20 and 50% of neurons unstained (Johnson et al. 1998; Young et al. 1993), when compared with immunohistological methods.

Inter-strain differences, such as those on pelvic innervation as described between Sprague-Dawley and Wistar rat strains by (Pascual et al. 1989) may contribute to the differences in neuronal density in the present and earlier studies. Using wholemount preparations of Sprague-Dawley ileum and antiserum against PGP 9.5, Johnson et al (1998) estimated the density of myenteric neurons to be 19600 ± 2030 per cm² in the 4 month rat ileum, whereas the present study would suggest there to be far fewer (between 12,000 and 13, 000 neurons/cm²) in the 6 month ileum.

From the measurements presented by Johnson et al, there appear to be substantial differences in small intestinal dimensions between the strains used; the Sprague Dawley small intestine appears to be shorter and wider than the Wistar-derived strain used in the present study. Inter-strain differences seem likely to account for the discrepancy between the present and previous studies, although as Johnson et al. did not discuss the degree of stretch of their tissue, it is unknown whether area determination has influenced the neuronal density they report.

Estimation of neuronal numbers in the enteric nervous system is thus clearly difficult, and comparison of the results of different studies is problematic. Study of developmental changes in neuronal numbers poses even more problems, as intestinal growth must be taken into account. Measurement of intestinal area, especially in young animals, introduces an additional potential source of error and the possibility of local differential growth cannot be discounted. Nevertheless, the results presented here indicate that neuronal numbers in the myenteric plexus increase between 7 and 14 days and between 21 and 28 days. This implies that “new” neurons are being added to myenteric ganglia after the intestine has become functional and critical for survival. This observation would suggest either that proliferation and differentiation of neuronal precursors is taking place in the postnatal rat myenteric plexus or that some partially differentiated neurons begin to express detectable levels of PGP 9.5 immunoreactivity after birth. Postnatal differentiation of precursors of neurons containing vasoactive intestinal peptide (VIP) and calcitonin gene related product (CGRP) has been reported in the murine enteric nervous system (Pham et al. 1991). Further work is needed to establish how the increase in neuronal numbers described in the present study is generated and to establish both the phenotype and location of such “new” neurons. The present observations, together with those of Pham et al (1991) on postnatal withdrawal of enteric precursors from the cell division cycle, and those on postnatal development of neurochemical phenotype (Faussone-Pellegrini et al. 1996; Matini et al. 1997; Vannucchi et al. 1997; Vannucchi and Faussone-Pellegrini 1996), indicate that enteric plexuses maintain a high degree of plasticity after the gut has become critical for survival. It is however, unclear how any “new” neurons would be integrated into existing neuronal circuits.

The results described here also indicate that a decrease in neuronal numbers may occur between 14 and 21 days, suggesting a loss of neurons from the myenteric plexus during this time. A period of naturally occurring cell death could be a possible explanation for the observed decrease in neuronal numbers. In other parts of the nervous system, a period of naturally occurring cell death commences shortly after target innervation is complete and is used to remove any inappropriate or excess connections by activation of a suicide pathway, often as a failure to receive limited amounts of a target-derived factor (as described in sections 1.6 and 1.7).

Naturally occurring cell death is difficult to study in the enteric nervous system, partially due to the existence of such complex microcircuits and the speed with which dying cells are removed, therefore at present, the occurrence of such a period has not been demonstrated in the enteric nervous system. An alternative explanation for the apparent decrease could be that myenteric neurons are migrating to give rise to part of the submucous plexus (Kapur et al. 1992; Pham et al. 1991). The observation that the submucous plexus develops both functionally and structurally later than the myenteric plexus (Faussone-Pellegrini et al. 1996; Faussone-Pellegrini et al. 1999; Matini et al. 1997; Vannucchi et al. 1997; Vannucchi and Faussone-Pellegrini 1996), would be consistent with this idea. Further investigation is necessary to fully characterise and determine the mechanism of the observed decrease.

The observed changes in neuronal numbers described in this study appear to centre around the time of weaning. Weaning was taken as occurring at 21 days, which was when the rat pups were removed from their mother and ceased to have access to milk. Suckling pups (7 and 14 days) had free access to laboratory chow and it is probable that they had begun to eat solid food prior to removal from their mother, although autopsy failed to reveal the presence of solid food in rat pup stomach contents prior to 14 days.

The stimuli for the observed changes in neuronal numbers are unknown, although some possibilities are suggested below;

- 1 The action of nutrients obtained from the diet which could act either directly after being absorbed through the gut, or indirectly by acting on other systems such as through a hormonal route.
- 2 Non-local action such as hormonally mediated effects that could be associated with developmental changes in other regions of the animal, influencing enteric neurons either directly or indirectly.
- 3 Local stimulation from target cells, including smooth muscle, glial cells, mucosa or other neurons. Potential mechanisms could involve target-derived neurotrophic factors or activity dependent mechanisms.
- 4 Extrinsic projections to myenteric ganglia (sympathetic - from coeliac ganglia or parasympathetic - from vagus) may also provide signals that influence development.

Diet-related effects

The diet of the rat pup may contain molecules that could influence the number of enteric neurons. Diet-derived factors that may affect the postnatal development of myenteric neurons have not been described. However, evidence suggests that breast milk may contain growth factors that stimulate postnatal development of other cells of the intestine (Yamashiro et al. 1989), which may in turn influence enteric neuronal development. Other factors found in the diet such as glucocorticoids (Martin and Henning 1984) and polyamines (Capano et al. 1998; Loser et al. 1999) have been found to stimulate the growth of intestinal cells (specifically mucosal and smooth muscle cells), which could in turn influence enteric neurons, although the effects of such factors on the development of myenteric neurons have not been investigated.

Non-local effects

During the postnatal period, the rat pup grows substantially, as indicated by an approximately 10 fold increase in body mass. Such an increase in size is associated with substantial development of other body regions such as the musculature, cardiovascular system and other regions of the nervous system (such as sympathetic and parasympathetic systems which project to the small intestine), all of which could potentially be related to the development of the enteric nervous system, either directly or indirectly.

Local effects

Many myenteric neurons are involved with moving foodstuff along the gastrointestinal tract and the movement of solid food along through the gastrointestinal tract will certainly require more physical effort than the movement of liquid food. Previous reports suggest that gut smooth muscle may thicken prior to weaning in preparation for the increased demands of a solid diet see (Thompson and Keelan 1986) and the increase in intestinal circumference seen in the present study would be consistent with this. The correlation between smooth muscle thickness and neuronal numbers has led to suggestion that smooth muscle thickness may influence the size of the myenteric neuronal population (Gabella 1987; Gabella 1992; Gabella and Trigg 1984; See et al. 1990). Increased thickness of gut smooth muscle during postnatal development could therefore potentially stimulate an increase in the number of myenteric neurons, possibly by way of target-derived actions of gut smooth muscle. The precise nature of this relationship between smooth muscle and enteric neurons and possible mechanisms of its regulation however have yet to be established, although intestinal smooth muscle could potentially stimulate enteric neurons either via chemical (such as target-derived neurotrophic factors) or electrical mechanisms (such as activity-dependent electrical signals), or both.

It is as yet unknown whether myenteric neurons are stimulated by activity-dependent effects of smooth muscle, however the presence of a number of neurotrophic factors has been described in the gastrointestinal tract (Saffrey and Burnstock 1994) and several of these have been found to influence myenteric neurons *in vitro* (see section 6.1). Neurotrophic factors are known to be essential in the development of other regions of the nervous system (Barde 1988; Barde 1989; Davies 1994; Davies 1996, amongst many others), but as yet little is known about the nature and roles of neurotrophic factors in the developing enteric nervous system. This is discussed further in chapter 6.

Neuronal size

The present study showed a great variation in neuronal size at all ages, however the mean size of myenteric neurons increased during the postnatal period. Factors involved in this increase are unknown but may be similar to those influencing neuronal numbers.

In summary, this study has revealed that the rat pup increases approximately 10 fold in size between 7 days and adulthood. Small intestinal area also increases dramatically during this time. The distance between individual myenteric ganglia increased in a similar manner to the increase in small intestinal area. The present study also suggests that neuronal development may also be incomplete in the suckling rat and the full compliment of myenteric neurons may not be achieved until approximately 35 days after birth. The pattern of neuronal development has revealed major changes that appear to centre on weaning. The stimuli for these changes are unknown, although many concomitant maturational changes occur within other gut layers that could potentially influence the maturation of the myenteric plexus.

CHAPTER 4

ULTRASTRUCTURE OF THE DEVELOPING SMALL INTESTINE

4.1 Abstract

Work presented in the previous chapter revealed changes in neuronal numbers during the first postnatal month of life. Possible stimuli for the observed changes are unknown, however there is indirect evidence for a relationship between smooth muscle thickness and neuronal numbers. The aim of this investigation was to make a detailed study of the ultrastructure of the myenteric plexus and surrounding smooth muscle layers during the first postnatal month. The results show that the myenteric plexus was relatively well developed in the suckling rat, although was immature relative to the adult. The proportion of myenteric ganglia surrounded by a basal lamina exhibited an age-related increase, however in the adult a small proportion of the plexus (approximately 15%) was not surrounded by a basal lamina. Throughout postnatal development a dense neuropil was present and myenteric neurons exhibited a high variability of shape and size. Developmental changes were also observed in the longitudinal and circular smooth muscle. The mean number of muscle cell profiles in both muscle layers increased significantly between 7 and 14 days, the time when an increase in neuronal numbers is observed. A further significant increase was observed between 14 and 21 days. From 21 days onwards, there was no further increase in the number of muscle cell profiles in either muscle layer, however a significant increase in the thickness of both layers was observed. Also from this time, the boundary between individual muscle cell profiles became more obvious and appeared to be surrounded by a layer of lucent material, not unlike the basal lamina surrounding the myenteric plexus. Specialisations within the circular muscle, which were present in the same location as type III ICC, became apparent from the end of the first postnatal month. In summary, development of the ultrastructure of the myenteric plexus continues into the postnatal period and the adult level of maturity appears to be established at the end of the first postnatal month. Changes in the smooth muscle thickness and cell number were observed at times concomitant with previously reported changes in neuronal numbers.

4.2 Introduction

The ultrastructure of both embryonic and adult myenteric ganglia has been studied in detail in several species, however the ultrastructure of the postnatal intestine has received little attention. Enteric ganglia exhibit a number of features that make them unique among autonomic ganglia, as previously described in section 1.4. Myenteric ganglia are comprised of neurons and glial cells, although one class of interstitial cells of Cajal (ICC) are associated with myenteric ganglia, they lie outside the basal lamina that surrounds individual ganglia. The wide variety of neuronal phenotypes observed at the light microscope level is reflected in the types of neuronal profile at the electron microscope level. However, as described previously (1.4.1) no correlation has been found between neuronal subpopulations identified at the light microscope level and neuronal profiles identified at the electron microscope level, as there appear to be many more neurochemical phenotypes than neuronal profiles (Furness and Costa 1980; Llewellyn-Smith et al. 1983).

Previous studies have shown that development of the myenteric plexus is ongoing through the first month of postnatal life, however ultrastructural changes have not previously been examined during this time. Evidence from work presented earlier in this thesis (see section 3.3) suggests that the myenteric plexus undergoes substantial changes during the postnatal period. Individual myenteric ganglia become more discrete, with the distance between them increasing significantly. Neuronal numbers during this time also change, with both significant increases and a decrease in neuronal numbers being observed. The increase in neuronal numbers between 7 and 14 days and between 21 and 28 days could suggest the presence of undifferentiated precursors, as previously discussed. The decrease in neuronal numbers between 14 and 21 days could suggest presence of dying neurons during this time.

Substantial increases in the length and the circumference of the small intestine were also observed during the postnatal period. The observed increase in small intestine circumference suggests that the muscularis externa is becoming thicker during this time. It is unknown whether the factors influencing small intestine growth are the same as those influencing the development of the myenteric plexus, or whether there is a relationship between the development of intestinal smooth muscle and the myenteric plexus. There is however indirect evidence to suggest a correlation between smooth muscle layer thickness and neuronal numbers (Gabella 1992; Gabella and Trigg 1984; See et al. 1990).

The aim of the work presented in this chapter was to study the ultrastructure of the myenteric plexus and surrounding smooth muscle during this period of substantial change. The variety of neuronal and glial cell profiles, presence of a neuropil, presence of a basal lamina and smooth muscle layer thickness was used to assess maturity at several developmental ages. The materials and methods used in this study are described in section 2.4.

4.3 Results

4.3.1 General appearance of the muscularis externa

The myenteric plexus of the adult rat ileum was very similar in appearance to that previously described in a number of other mammalian species (Gabella 1987; Gabella and Trigg 1984). Adult myenteric ganglia appeared to be compact, packed with a dense neuropil and to contain only neuronal and glial elements. A basal lamina was clearly visible around most myenteric ganglia. Ultrastructural descriptions of myenteric ganglia in the adult intestine suggest that all myenteric ganglia are surrounded by a basal lamina. The results presented in this study suggest that although the majority (85%) of adult ganglia are surrounded by a basal lamina, a proportion of the myenteric plexus remains in direct contact with smooth muscle cells and connective tissue. The presence of interstitial cells of Cajal (ICC) during the postnatal period was not systematically examined in the present study, although they are known to be present in the intestine at birth (Faussone-Pellegrini et al. 1996; Vannucchi et al. 1997).

Evidence from work presented earlier in this thesis indicates the addition of “new” neurons to the myenteric plexus, possibly suggesting the presence of dividing undifferentiated precursors in the myenteric plexus during postnatal development (see section 3.3.4). Cells with the appearance of those undergoing cell division were not detected in myenteric ganglia during the present study. However, in order to conclude that dividing cells were not present within myenteric ganglia during postnatal development, much more tissue would need to be analysed. The decrease in neuronal numbers observed between 14 and 21 days (see section 3.3.4) may suggest the presence of dying neurons between 14 and 21 days. Although this study was not exhaustive, myenteric ganglion cells exhibiting the characteristic “chromatin blebbing” of dying cells was not detected, possibly because the breakdown and removal of such cells occurs rapidly.

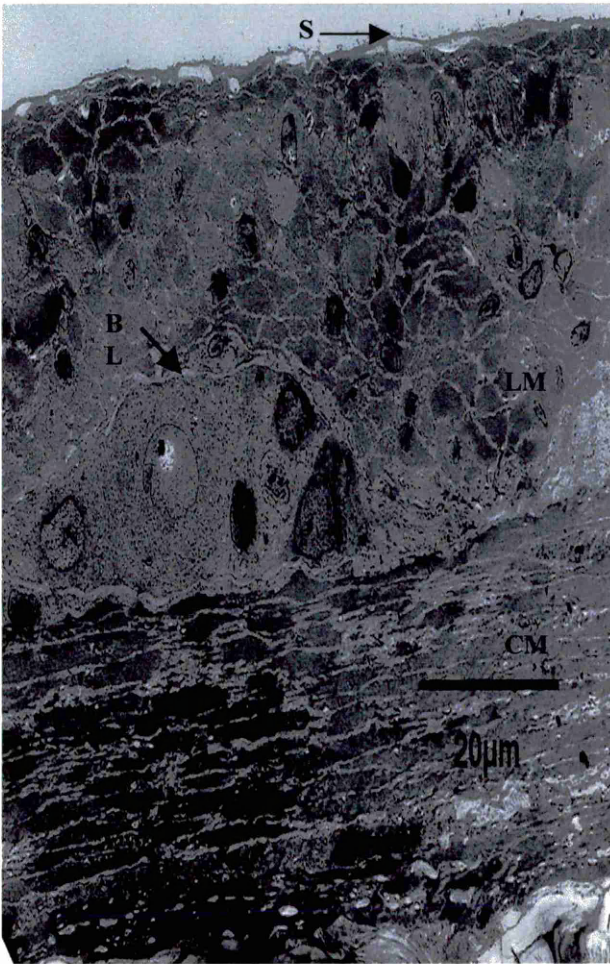
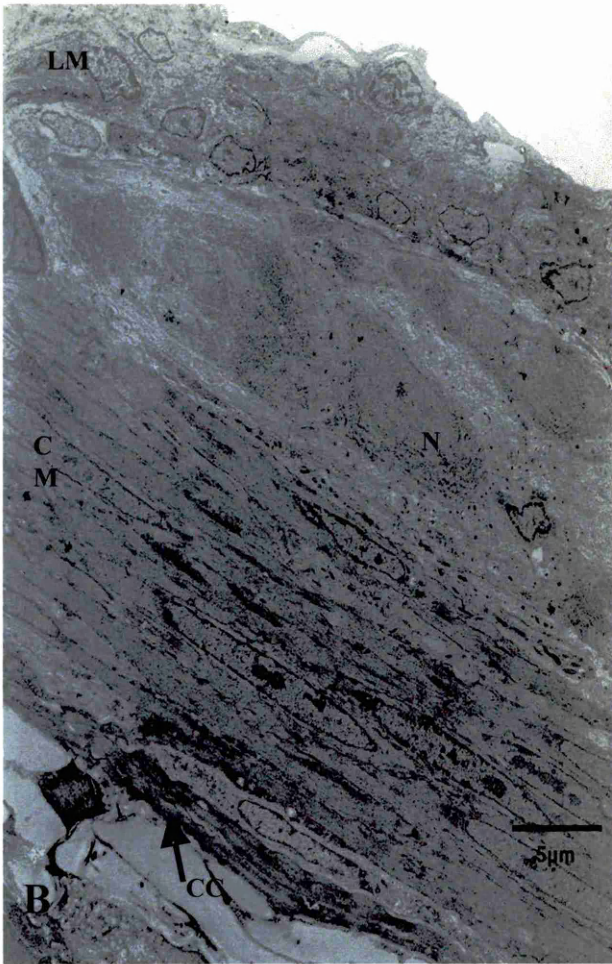
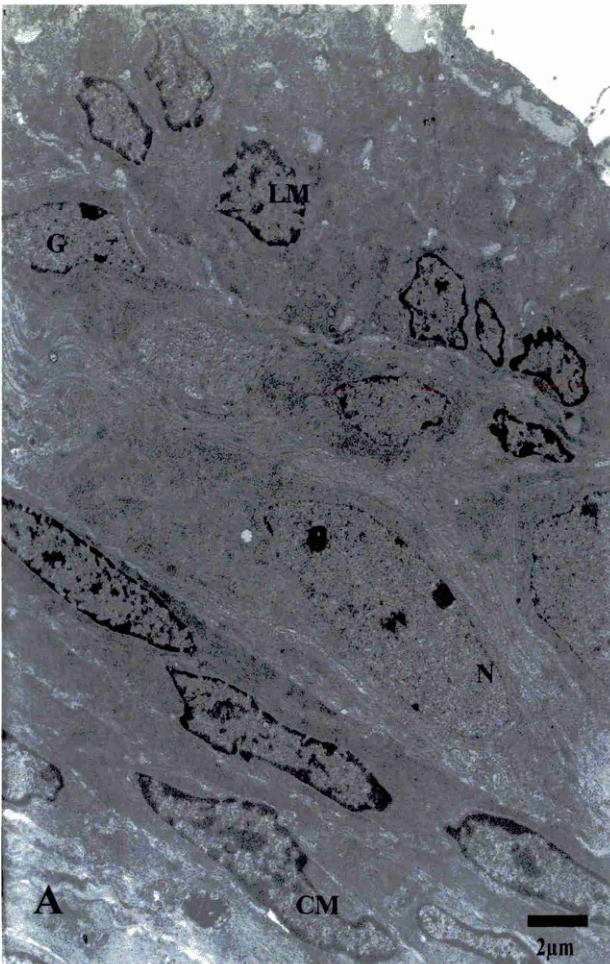
The myenteric plexus was well developed and myenteric ganglia contained a dense neuropil at all of the ages studied (see figure 4a). Myenteric ganglia in the suckling rat (7 and 14 days) appeared to be more densely and more closely packed than at later ages. This is consistent with observations at the light microscope level (see section 3.3.4). A basal lamina was present in the younger animals (7 and 14 days), but appeared to be much less prominent than in older animals. The proportion of ganglia surrounded by a basal lamina exhibited an age-related increase during the postnatal period (figure 4b). In the 7 day rat pup, approximately 35% of myenteric ganglia were surrounded by a basal lamina, whereas in the 21 day rat pup coverage of ganglia by a basal lamina had increased to approximately 60%. A further increase in the proportion of ganglia surrounded by a basal lamina was observed between the end of the postnatal month and adulthood.

4.3.2 Ultrastructural appearance of myenteric neurons

Myenteric neurons had a relatively mature appearance at 7 days and exhibited an array of shapes and sizes similar to that observed in the adult. As in the adult, nuclei were often oval with few, shallow indentations to the nuclear envelope and with between 0 - 3 nucleoli. The variety of neuronal profiles remained obvious throughout development, although in general they became a little larger.

Cytoplasmic organelles were abundant throughout development, the most prominent being mitochondria and ribosomes. Figure 4c shows a typical adult myenteric neuron and figure 4d illustrates neuronal profiles at different postnatal ages. The variety of neuronal shapes and sizes that were observed is consistent with observations at the light microscope level and as reported in earlier ultrastructural studies of the guinea pig.

Figure 4a Low magnification electron micrographs of myenteric ganglia during postnatal development



A Section through a 7 day ileum wall. Note the lack of definition of individual muscle cells and a basal lamina

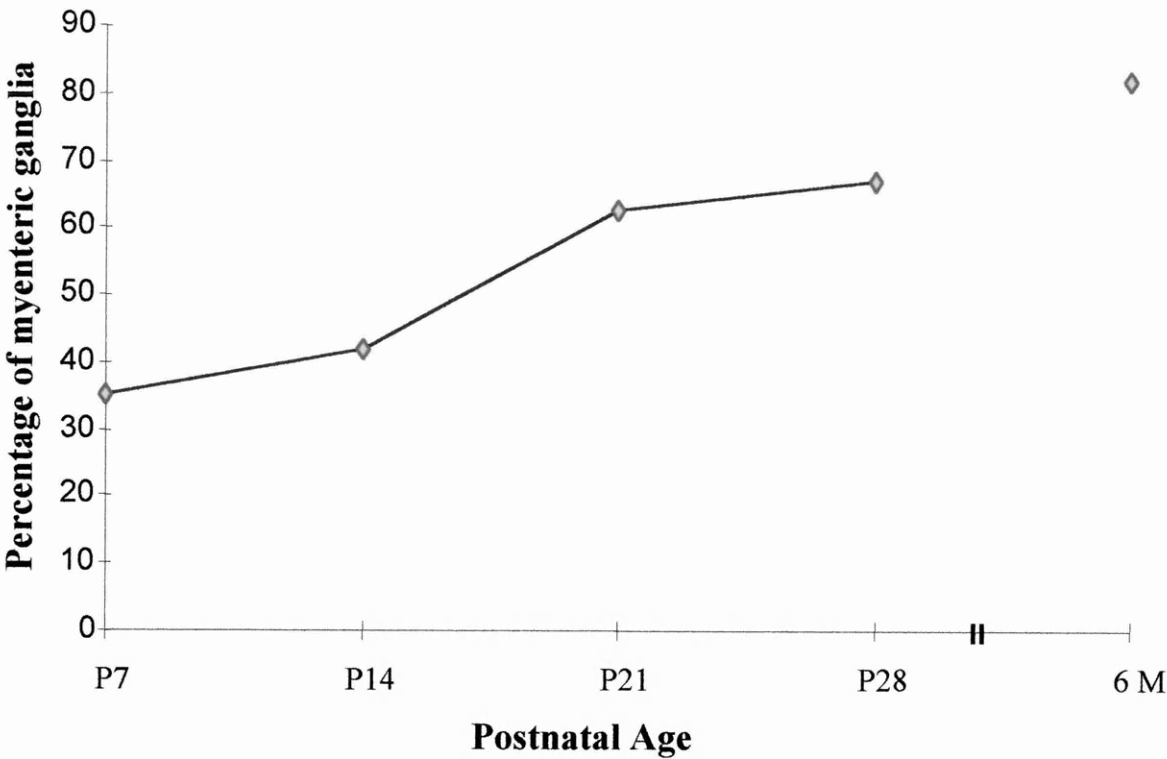
B Section through a 21 day ileum wall. Note specialisations within the circular muscle. Basal lamina still unclear.

C Section through a 28 day ileum wall. Note the lucent layer surrounding individual muscle cells. Part of a basal lamina is also visible.

Abbreviations

BL	Basal lamina
CC	Condensed muscle cell
CM	Circular muscle cell
CT	Connective tissue-like layer
G	Glial cell
LM	Longitudinal muscle cell
N	Neuron
S	Serosa

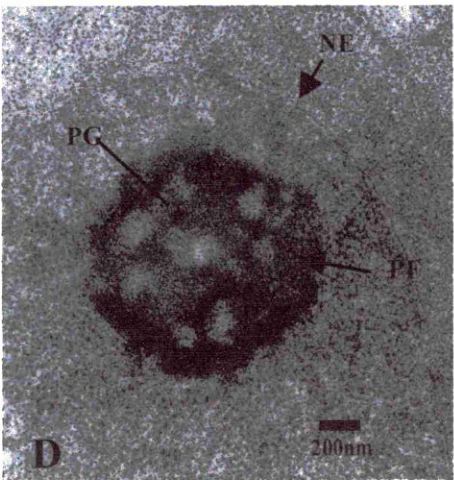
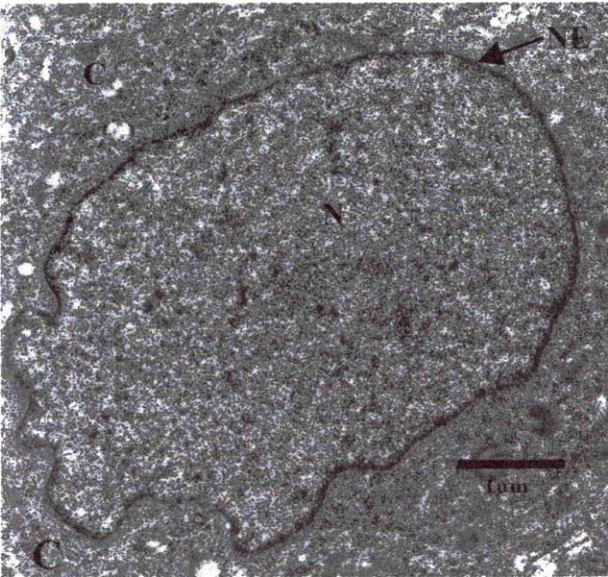
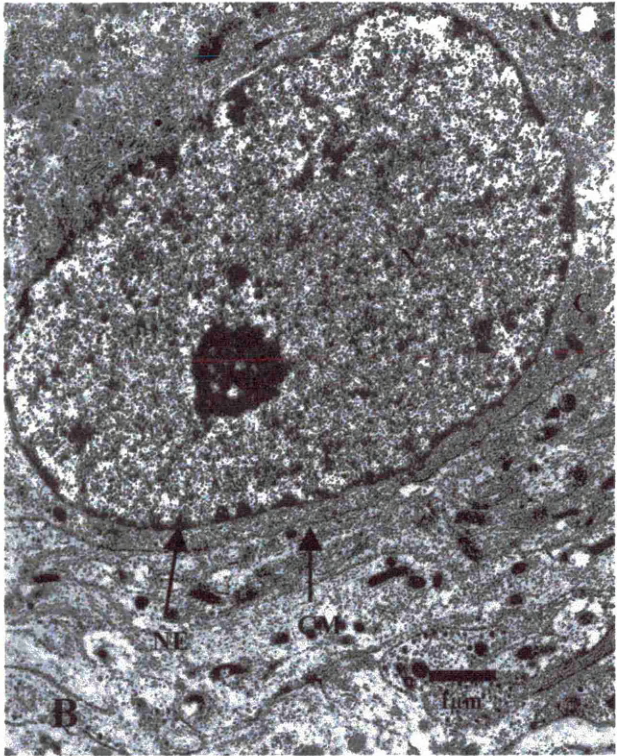
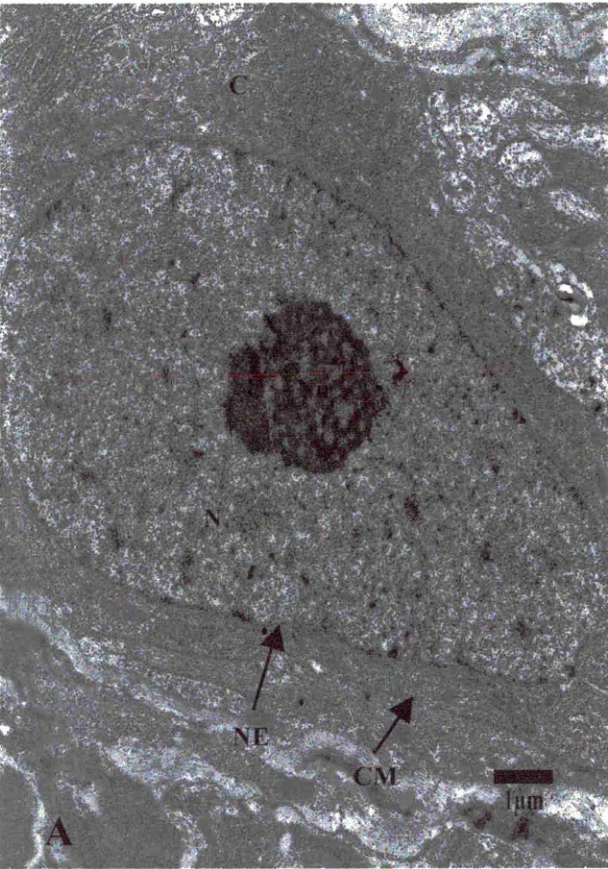
Figure 4b Age-related increase in the proportion of myenteric ganglia surrounded by a basal lamina



The number of myenteric ganglia surrounded by a basal lamina was recorded at each age and calculated as a percentage of the total number of myenteric ganglia. As the scale is discontinuous between 28 days and 6 month, the points are not joined. P denotes postnatal days and M denotes postnatal months.

P7	n=3
P14	n=3
P21	n=5
P28	n=3
6M	n=3

Figure 4c **High magnification electron micrographs of neuronal profiles in the adult myenteric plexus**



A and B
Neuronal profiles containing a single nucleolus.

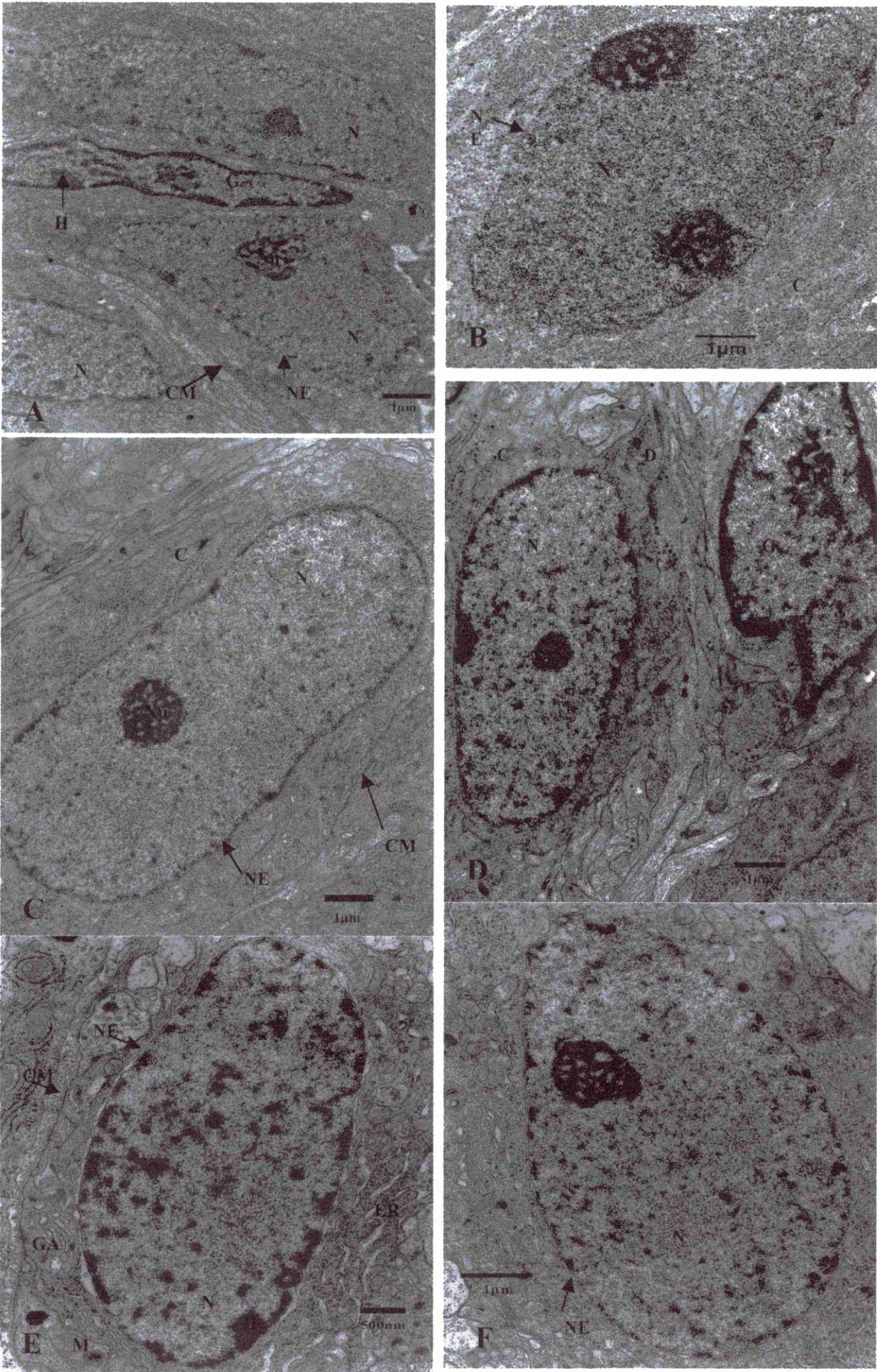
C Neuronal profile with no apparent nucleolus.

D Typical nucleolus at higher magnification.

Abbreviations

NE	Nuclear envelope
Nu	Nucleolus
C	Cytoplasm
N	Nucleoplasm
PG	Pars granulosa
PF	Pars fibrosa
CM	Cell membrane

Figure 4d **High magnification electron micrographs of neuronal profiles in the developing myenteric plexus**



4.3.3 Ultrastructural appearance of enteric glial cells

Glial cell size and shape varied greatly throughout development. It is well documented that some enteric glial cells resemble central nervous system astrocytes (Gabella 1981; Gabella and Trigg 1984; Jessen and Mirsky 1980; Jessen and Mirsky 1983), and the majority of glial cells observed in this study resembled one of two astrocyte-like profiles.

Cytoplasmic organelles were relatively sparse, although mitochondria and gliofilaments were prominent in both profile types. Fibrous-astrocyte-like profiles exhibited a thin layer of heterochromatin beneath the nuclear envelope and heterogeneous nucleoplasm, whereas protoplasmic-astrocyte-like profiles exhibited dense condensations within the nucleoplasm as well as beneath the nuclear envelope. From 21 days onwards, glial cell profiles with “teardrop” shaped nuclei became apparent. Cells of this type exhibited features similar to both types of astrocyte-like profiles. Figure 4f illustrates some of the glial cell profiles observed during this study.

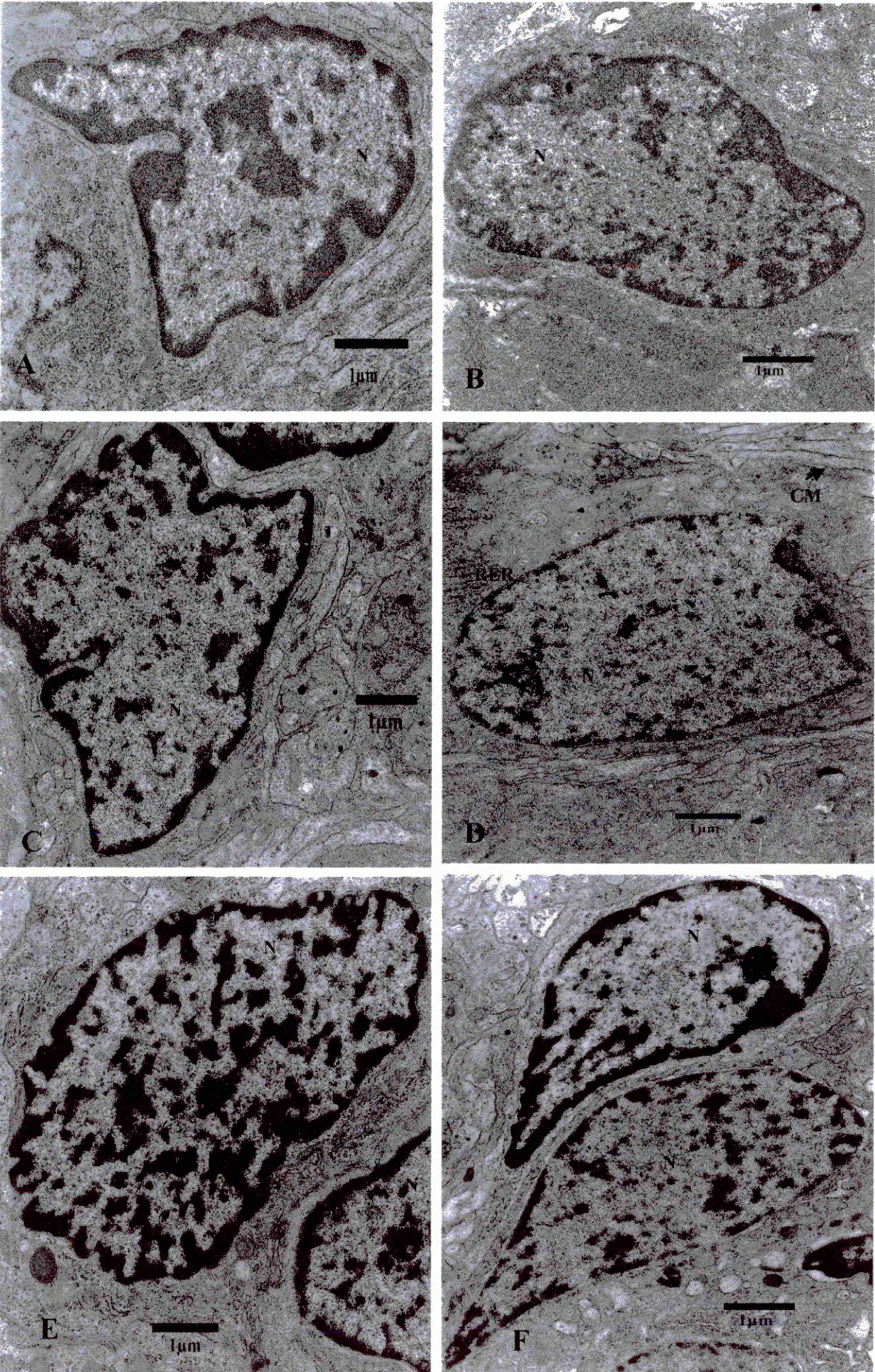
**Figure 4e High magnification electron micrographs of glial cell profiles in the
developing myenteric plexus**

- A and B Glial cell profiles from 7 day myenteric ganglia. Note the variation of size, shape and heterochromatin distribution.
- C and D Glial cell profiles from 14 day myenteric ganglia. C is an example of a fibrous astrocyte-like glial cell. D is an example of a protoplasmic astrocyte-like glial cell.
- E and F Glial cell profiles from 21 day myenteric ganglia. E is an example of a fibrous astrocyte-like glial cell. F shows the teardrop shaped glial cells which became obvious from 21 days onwards.

Abbreviations

- N Nucleoplasm
CM Cell membrane
H Heterochromatin
NE Nuclear envelope
RER Rough endoplasmic reticulum

Figure 4e **High magnification electron micrographs of glial cell profiles in the developing myenteric plexus**



4.3.4 Ultrastructural appearance of intestinal smooth muscle

Longitudinal muscle cells were cut in transverse section and were irregular in appearance. The nuclei of longitudinal muscle cells were irregular in appearance, with homogeneous nucleoplasm and electron dense condensations beneath the nuclear envelope. Circular muscle cells appeared rod-like and were more regular in shape. Muscle cells exhibited elongated nuclei, homogeneous nucleoplasm and electron dense condensations beneath the nuclear envelope. Filaments of various thickness were also observed, as illustrated in figures 4h and 4i. Such filaments were more prominent in the circular than in the longitudinal muscle due to the orientation of the specimen. Smooth muscle cell profiles observed in the developing and adult intestine are shown in figures 4h and 4i. Until 21 days, it was difficult to distinguish the boundaries of individual muscle cells, although their nuclei were easily identifiable. In the 21 day ileum, the border of individual muscle cells appeared to become outlined by a lucent lamina-like material which had a similar appearance to that of connective tissue. This appeared thicker and more granular from 21 days onwards. Also from 21 days, specialisations become apparent within the innermost part of the circular muscle - the 2 or 3 cells closest to the submucosa appeared more electron dense and were shortened and overlapped (see figure 4a, image B). These cells were in the location where type III ICC have been described, however it is unknown whether these cells were ICCs or smooth muscle cells so for the purpose of muscle thickness analysis, these cells were excluded. Immunohistochemistry for c-kit could be used to establish the nature of these cells.

The thickness of the longitudinal and circular muscle layers was measured at each age and the mean muscle thickness calculated (see figure 4f). At 7 days the mean thickness of the longitudinal muscle was $12.32 \pm 0.72\mu\text{m}$ and the mean thickness of the circular muscle was $18.49 \pm 0.96\mu\text{m}$.

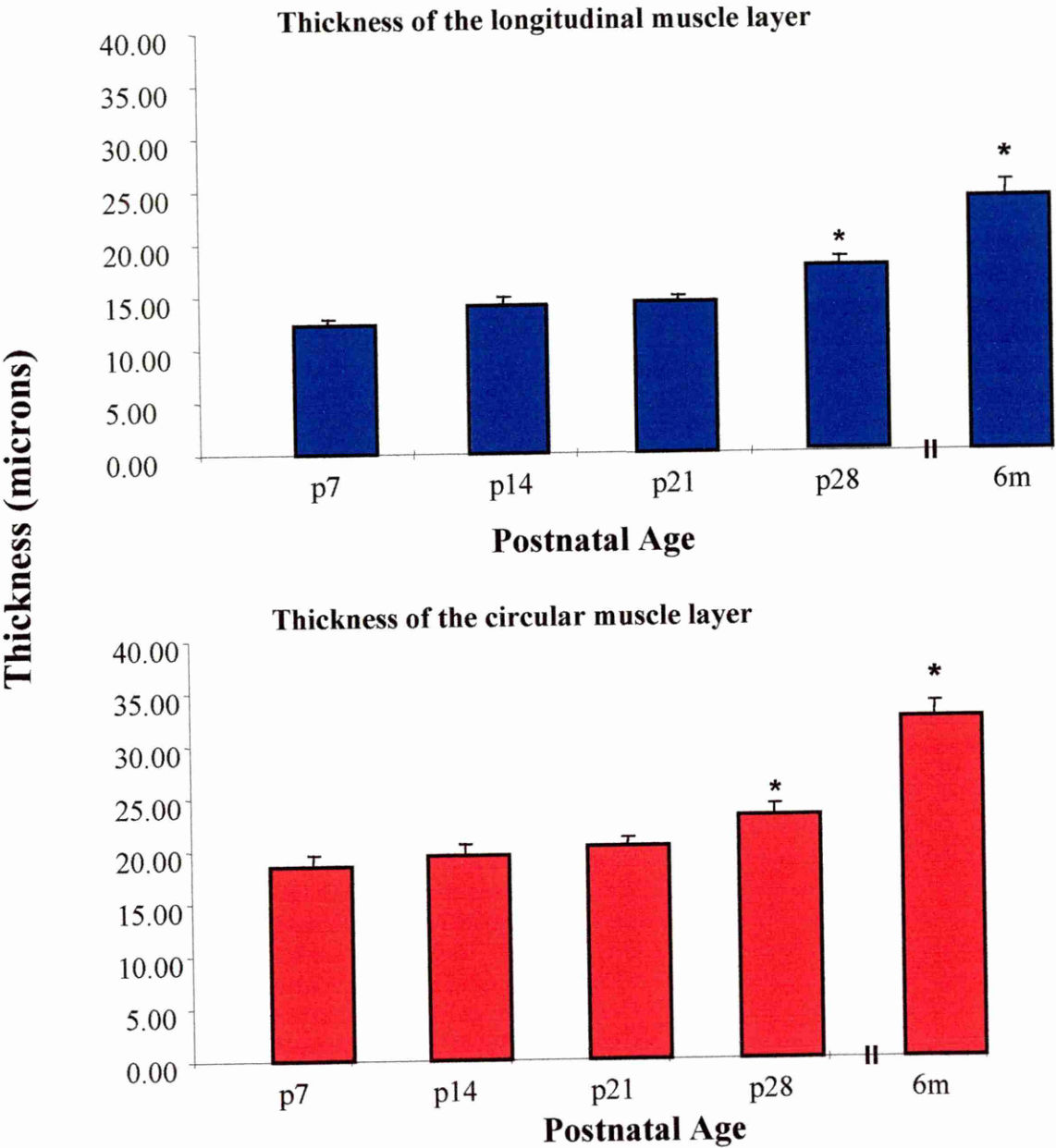
At each age examined, the circular muscle layer was consistently thicker than the longitudinal muscle (see figure 4g). The results show that there is no significant difference between the thickness of either muscle layer during the first 3 weeks of postnatal life (one way Anova, $p < 0.01$). Between 21 and 28 days the mean thickness of the longitudinal muscle (to $17.50 \pm 0.70 \mu\text{m}$) and the circular muscle increased significantly (to $23.17 \pm 1.00 \mu\text{m}$). Further significant increases were observed in both muscle layers between 28 days and adulthood, the mean longitudinal muscle thickness reaching $24.01 \pm 1.53 \mu\text{m}$ and the circular muscle thickness reaching $31.24 \pm 1.54 \mu\text{m}$.

Previous attempts at assessing intestinal smooth muscle thickness in the adult have not considered the effects of tissue shrinkage during preparation for electron microscopy, which can be substantial in some tissues (King 1991; Uylings et al. 1986). Shrinkage effects were not assessed in the present study and it is unknown whether small intestine samples of different ages will be differently affected, so the number of muscle cell profiles within each muscle layer was compared as another measure of muscle layer thickness.

The number of muscle cell bodies was recorded across the thickness of both the longitudinal and circular muscle layers throughout postnatal development. The mean is presented with the standard error for each age group (figure 4g). Statistical analysis (Anova, $p < 0.01$) revealed a significant increase in the mean number of muscle cell profiles in the longitudinal muscle layer between 7 and 14 days (from 4.93 ± 0.19 to 6.62 ± 0.21) and between 14 and 21 days, reaching 8.03 ± 0.18 muscle cell profiles. There was no significant difference between the number of muscle cell profiles between 21 days and adulthood.

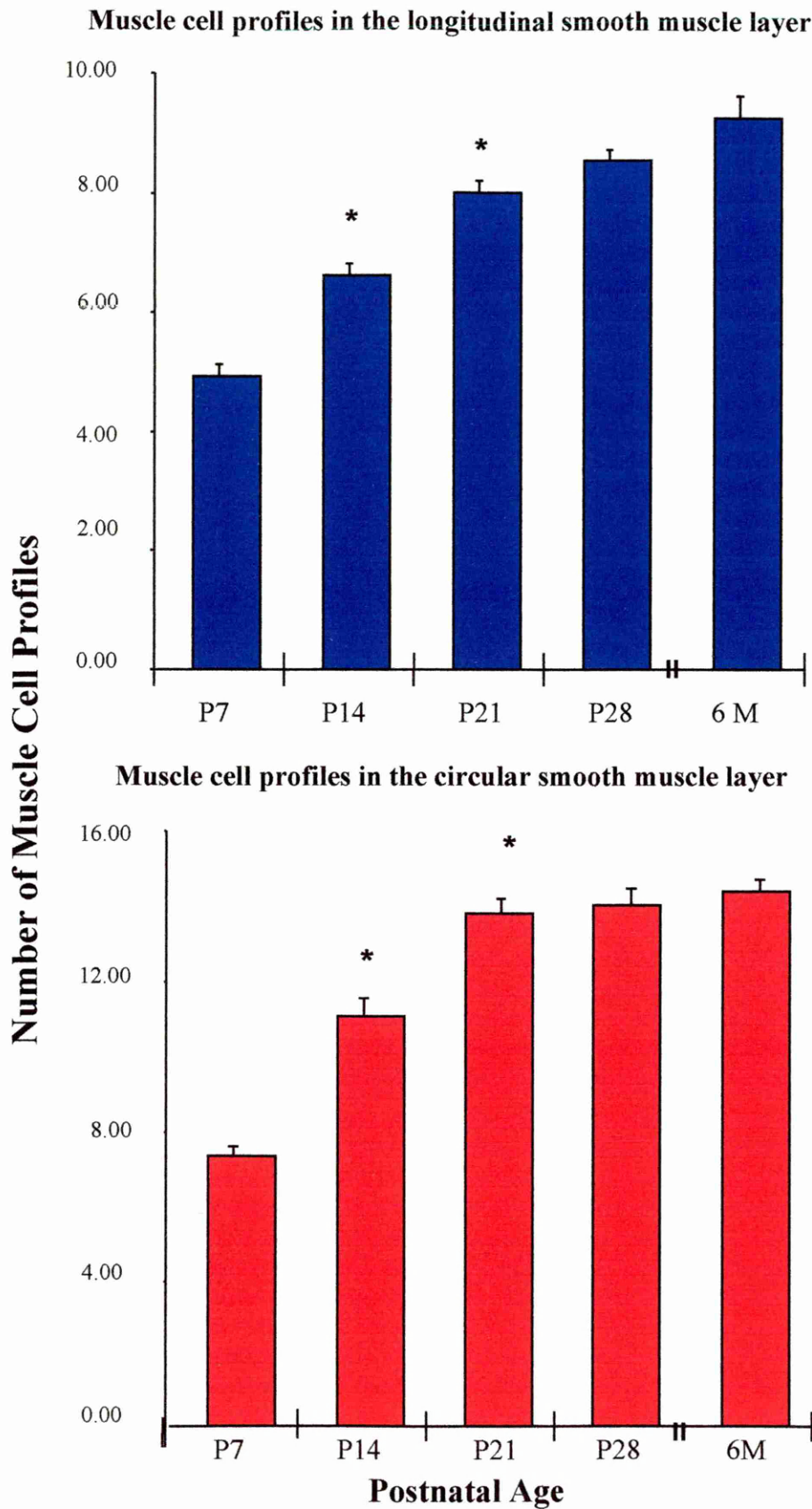
The mean number of muscle cell profiles across the circular muscle also increased significantly between 7 and 14 days (from 7.17 ± 0.23 to 10.86 ± 0.46). A further significant increase was observed between 14 and 21 days to reach 13.55 ± 0.41 muscle cell profiles. As observed in the longitudinal muscle, there was no significant increase in the number of muscle cell profiles between 21 days and adulthood, however at all of the ages examined, the circular muscle layer was consistently thicker than the longitudinal muscle (see figure 4g).

Figure 4f **Changes in the thickness of the smooth muscle layers of the muscularis externa during postnatal development**



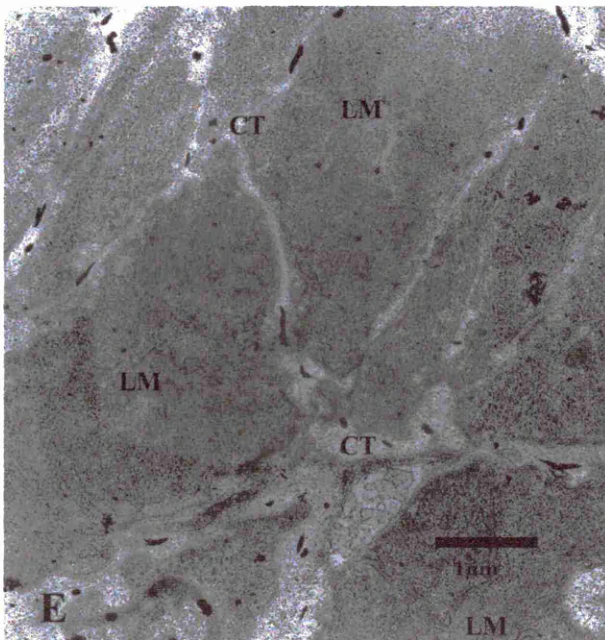
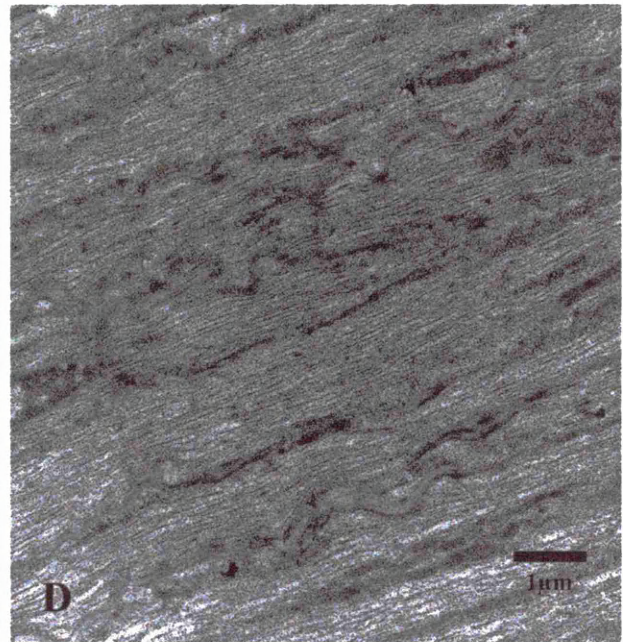
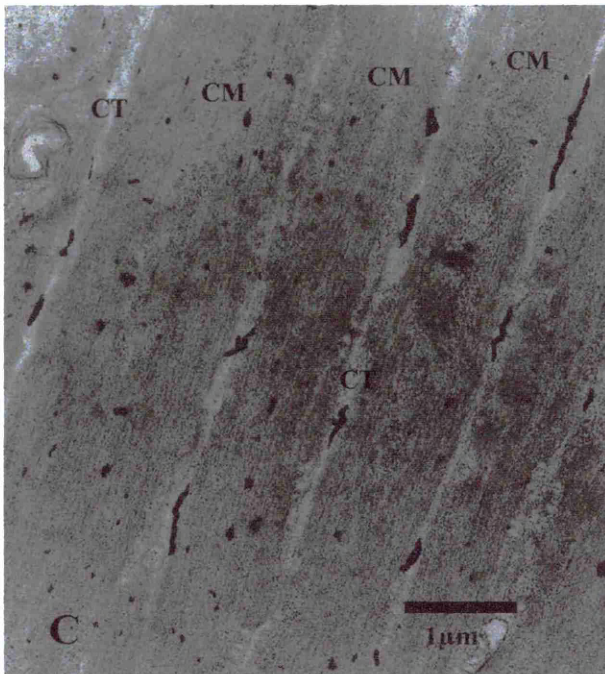
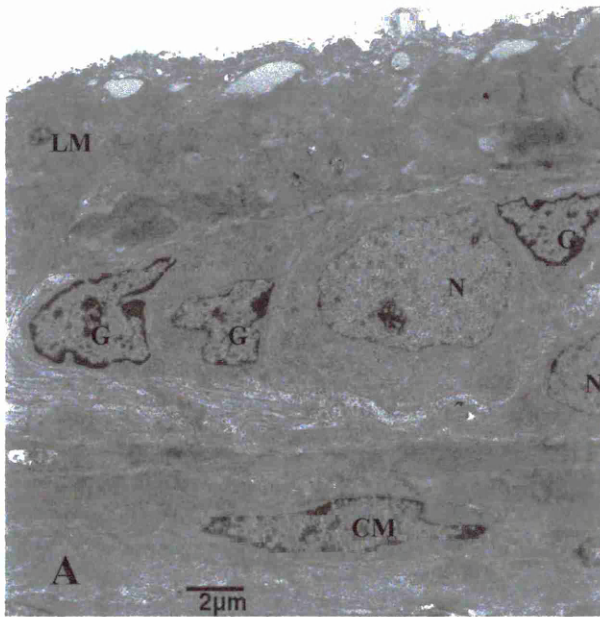
The thickness of the longitudinal and circular smooth muscle layers was measured at different ages during postnatal development. The mean is plotted for each age group. P denotes postnatal days and M denotes postnatal months. * indicates significant ($p<0.05$) difference from muscle layer thickness at previous ages.

Figure 4g **Changes in the number of muscle cell profiles within the muscularis externa during postnatal development**



The mean number of muscle cell profiles is plotted +/- the standard error for each age group. * indicates significant ($p<0.05$) difference from the number of profiles at previous ages. Thirty measurements were made in each case. P denotes age in postnatal days whereas M denotes age in postnatal months.

Figure 4h Muscle cell profiles in the rat ileum during postnatal development



A Section through a 7 day ileum. Note the lack of definition of individual muscle cells, although muscle nuclei are prominent.

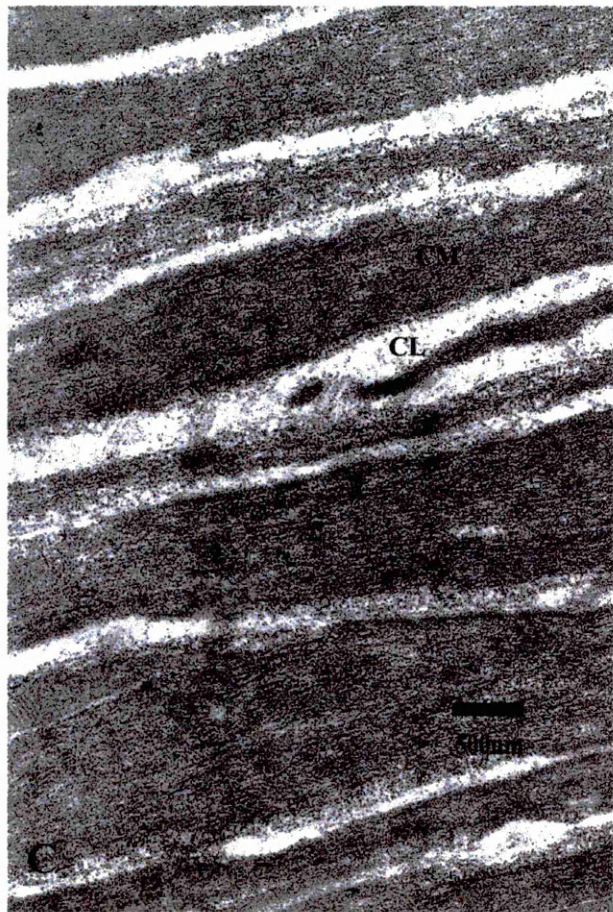
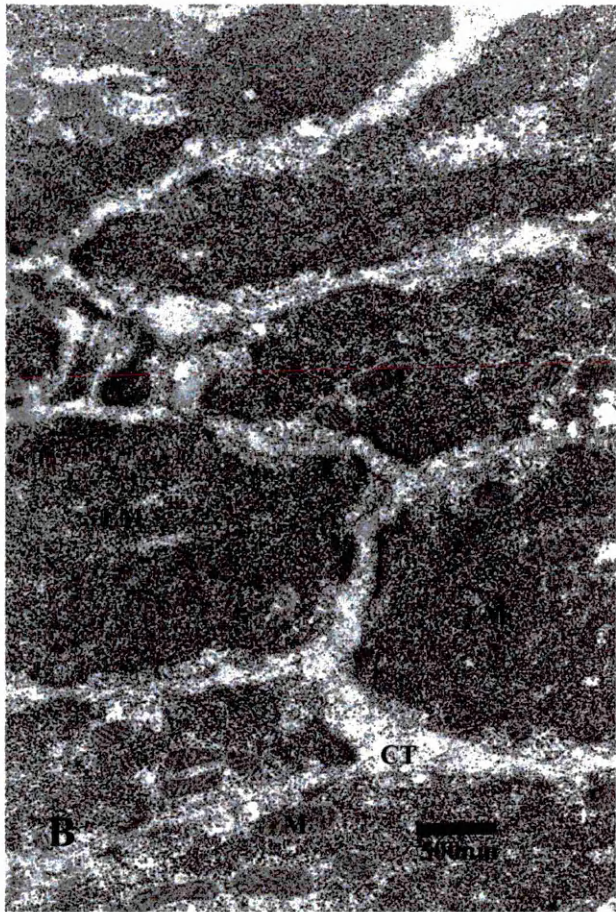
B Section through a 14 day ileum. Note the lack of definition of individual muscle cells, although muscle nuclei are prominent.

C Section through 21 day circular muscle. Note the connective tissue-like layer which surrounds individual cells.

D 21 day circular muscle at higher magnification. Filaments of various thickness are distinguishable.

E Section through 21 day longitudinal muscle. Note the connective tissue-like layer which surrounds individual cells.

Figure 4i **High magnification electron micrographs of muscle cells profiles in the adult rat ileum**



A Part of a myenteric ganglion surrounded by longitudinal and circular muscle cells. Note the basal lamina separates the ganglion from the muscle layers.

B Longitudinal muscle cells. Note the abundance of mitochondria and the presence of a granular connective-tissue like sheath around individual cells.

C Circular muscle cells. Note the presence of a granular connective-tissue like sheath around individual cells.

Abbreviations

N	Neuronal nucleus
CM	Circular muscle cell
LM	Longitudinal muscle cell
M	Mitochondria
CT	Connective tissue-like layer

4.4 Discussion

Although the ultrastructure of the adult enteric nervous system has been well described, ultrastructural studies of the developing enteric nervous system, particularly during the postnatal period, are few. The aim of this investigation was to make a detailed study of the myenteric plexus of the rat small intestine during the first month of postnatal life.

In the adult, myenteric ganglia are surrounded by a basal lamina that separates them from the surrounding smooth muscle and connective tissue. The basal lamina was found to be developmentally acquired, as described in earlier studies in the guinea pig small intestine (Gabella 1982). It is unclear how interactions between smooth muscle and myenteric ganglia change as the basal lamina forms. Interestingly, a small proportion of adult myenteric ganglia appeared to be in direct contact with smooth muscle cells, as a basal lamina was only apparent around approximately 85% of the myenteric plexus. Direct contact between enteric ganglion cells and longitudinal smooth muscle has been reported previously in the rat small intestine (Komuro 1988). The possible role of such direct contact between enteric neurons or glial cells and smooth muscle cells is uncertain, however the observation from the present study that there is a progressive decrease in the number of ganglia in direct contact with smooth muscle, may indicate a change during postnatal development. Contact between smooth muscle and enteric ganglia may act to anchor the smooth muscle to the ganglia in order to maintain relative positions during muscular contraction. It has also been suggested that, as the longitudinal muscle in many regions of the gut is only sparsely innervated by nerve fibres, neurotransmitters released from the myenteric plexus may reach the muscle by diffusion. It is also possible that direct contact may allow ganglion cells access to molecules and to electrical signals that could act as neurotrophic factors, although at present there is no evidence to support this suggestion.

In the 7 day rat ileum, myenteric ganglia were relatively well developed, gradually becoming more discrete and compact as development progressed. A dense neuropil was present throughout development. A variety of neuronal shapes and sizes were observed at this age and although in general neurons appeared to increase slightly in size during development, a similar variety of profiles were observed throughout development. Neurons were easily distinguished from glial cells by their nuclei, as observed in several mammalian species (Cook and Burnstock 1976; Gabella 1972; Gabella 1981; Gabella 1987; Gabella and Trigg 1984). Neuronal nuclei were large, eccentrically positioned and had a smooth nuclear envelope, whereas glial cell nuclei were irregular in shape, contained an abundance of condensed chromatin and contained more granular nucleoplasm.

Enteric glial cells are unique among peripheral glia and this unique nature may be important in conferring structural support to myenteric ganglia, especially during the mechanical stress of muscular contraction but at the same time allowing structural rearrangement. (Gabella 1981; Gabella 1990; Wilson et al. 1981). Enteric glial cells have also been found to exhibit characteristics of CNS astrocytes (Gershon and Rothman 1991; Jessen and Mirsky 1980; Jessen and Mirsky 1983). In the present study, glial cells morphologically similar to fibrous and protoplasmic CNS astrocytes were observed throughout development, however glial cell profiles exhibited such a wide variety of shapes and sizes, that systematic classification was not possible. Glial cells that were “teardrop shaped” were observed from 21 days onwards, although it is possible that this type of glial cell was present at previous ages. Observations from the present study are in agreement with earlier studies on the guinea pig (Gabella and Trigg 1984).

Work presented previously in this thesis revealed an increase in neuronal numbers between 7 and 14 days and between 21 and 28 days (see section 3.3.4). The stimulus for this increase is unknown, however a relationship between the smooth muscle layer and neuronal numbers has been suggested (Gabella 1992; Gabella and Trigg 1984; See et al. 1990) and several neurotrophic factors have been shown to be present in intestinal smooth muscle (see table 7). Possible changes in smooth muscle thickness and muscle cell number were examined in relation to the observed increases in neuronal number in order to assess whether there may be a relationship between their development.

The number of muscle cell profiles increased significantly between 7 and 14 days, which correlates with the increase in neuronal numbers. There was however no increase in muscle cell profiles during the second period of neuronal number increase (between 21 and 28 days). However there appeared to be a correlation between the increase in neuronal numbers between 21 and 28 days and the increase in the thickness of the intestinal smooth muscle at that time, however the recorded changes in smooth muscle thickness ought to be treated with caution as possible effects of tissue shrinkage are uncertain. It is unknown whether the factors that influence the development of intestinal smooth muscle are the same as those that influence the development of the myenteric plexus or whether one regulates the development of the other.

The decrease in neuronal numbers between 14 and 21 days did not appear to correlate with changes in intestinal smooth muscle ultrastructure, however the possibility of other changes within the smooth muscle, such as chemical changes, cannot be ruled out. The influence of intestinal smooth muscle on enteric neuronal development is unknown but may be considerable; it is important to study the developing smooth muscle as it could be a source of target derived neurotrophic factors that may influence the development and maintenance of myenteric neurons.

As mentioned previously, the number of muscle cell bodies significantly increased in both smooth muscle layers until weaning (21 days), when the number of muscle cell profiles appeared to stabilise in each layer. Also at this time, a number of specialisations became apparent within the inner circular muscle layer. These specialisations, a thickening of the innermost cells, were widespread from the end of the first postnatal month of life. The specialised cells appear in the same location as type III interstitial cells of Cajal (ICC). It is unknown whether these cells were ICC's or smooth muscle cells, however immunohistochemistry for c-kit could be used to establish the nature of these cells.

The boundary of individual muscle cell profiles was difficult to determine at lower magnifications until weaning. From weaning onwards, individual muscle profiles appeared to be surrounded by a lamina-like structure, such as that observed in the adult canine intestine (Henderson et al. 1971). Studies of the chicken and guinea pig gastrointestinal tract have led Gabella (1992) to suggest that intestinal smooth muscle cells express a homogeneous phenotype and develop simultaneously. It was difficult to determine whether intestinal smooth muscle cells expressed a homogeneous phenotype in this investigation, although in the adult, there appeared to be muscle cell profiles exhibiting a range of electron densities within both muscle layers.

In summary, the appearance of the adult rat myenteric plexus is similar to that of the guinea pig. Although the 7 day myenteric plexus is relatively well developed, the adult level of maturity does appear to be achieved until post weaning. The basal lamina that surrounds the adult myenteric ganglia appears to be developmentally acquired, as described previously for the guinea pig, however a proportion of adult myenteric ganglia appear to remain in direct contact with smooth muscle. A similar range of neuronal and glial profile shapes and sizes was observed throughout development, the exception being teardrop shaped glial cells which became apparent from weaning onwards.

Developmental changes were also observed in the intestinal smooth muscle. The number of muscle cells significantly increased in both smooth muscle layers until weaning and then appeared to stabilise. Smooth muscle layer thickness appeared to increase from weaning onwards, at the same time as specialisations within the inner circular muscle layer became apparent. These observations suggest that increase in neuronal numbers between 7 and 14 days may be associated with an increase in the number of smooth muscle cells and that the increase in neuronal numbers between 21 and 28 days may be associated with increases in smooth muscle thickness. At the time when a decrease in neuronal was observed (between 14 and 21 days), the number of muscle cells ceased increasing and muscle cell thickness began to increase.

CHAPTER 5

DEVELOPMENT OF NADPH-DIAPHORASE POSITIVE MYENTERIC NEURONS

5.1 Abstract

The aim of this study was to investigate possible postnatal changes in the nitric oxide (NO) synthesising subpopulation of myenteric neurons in the rat ileum. Following paraformaldehyde fixation, neurons containing nitric oxide synthase (NOS) were visualised in wholemount preparations of ileum using the NADPH diaphorase histochemical method. The number of NADPH diaphorase positive neurons and their somal area was measured. The density of neurons containing the formazan reaction product were calculated and compared with that of the total number of myenteric neurons at each age (as determined by PGP 9.5 immunohistochemistry). In summary, the results show a dramatic increase in the numbers of nitric oxide synthesising myenteric neurons during the suckling period. The NADPH diaphorase positive population remained stable over the period of diet change from liquid to solid food, suggesting no role of dietary factors in density increase. A significant increase in neuronal number was observed between 28 days and 6 months, suggesting that the final complement of neurons expressing this phenotype is not achieved until after the end of the first postnatal month. The results also show that the mean size of NO synthesising neurons increases over 3 fold between the suckling period and adulthood.

5.2 Introduction

It is widely accepted that the action of a certain class of intrinsic myenteric neurons elicits relaxation of gastrointestinal smooth muscle. These neurons are commonly referred to as nonadrenergic-noncholinergic (NANC) neurons as they utilise neither noradrenaline nor acetylcholine as neurotransmitters. NANC nerves mediate most inhibitory responses of intestinal smooth muscle and regulate many important physiological reflexes such as relaxation of the lower oesophageal sphincter after swallowing, receptive relaxation of the proximal stomach during eating and descending inhibition in response to distension (see Sanders and Ward 1992). Until recently, the identity of the transmitter(s) employed by these nerves was elusive; the main candidate molecules being ATP and the peptide VIP (see Furness et al. 1992). There is now good evidence however, that nitric oxide (NO) is the major NANC transmitter in the mammalian GI tract (Murray et al. 1991; Nakamura et al. 1998; Sanders and Ward 1992; Shuttleworth et al. 1991; Toda et al. 1990).

Unlike many other transmitters, nitric oxide is a gas and is not stored in neurons, being synthesised from L-arginine prior to release via a calcium/calmodulin dependent mechanism (Vincent and Hope 1992). Neurons which utilise NO as a transmitter have been identified predominantly using two techniques; immunohistochemistry for the enzyme which mediates the synthesis of NO, nitric oxide synthase (NOS) or by a histochemical reaction in which the NADPH-diaphorase activity of NOS (Hope et al. 1991) is used to convert a soluble tetrazolium salt to an insoluble, visible formazan (Scherer-Singler et al. 1983; Vincent et al. 1986).

The NADPH diaphorase reaction has been reported only to identify NOS that constitutively synthesises NO in neurons (Type I or nNOS) and not NOS that is inducible (Type II) or NOS found in endothelial cells (Type III) (see Grozdanovic et al. 1992).

However, there is some evidence to suggest that this may not be the case in all tissues as NADPH diaphorase activity has been shown in the epithelial cells of the neonatal guinea pig urinary bladder (Saffrey et al. 1994) and non neuronal cells in the kidney, lung, uterus, and stomach (Schmidt et al. 1992). Specific antisera that recognise only neuronal NOS are also now available although to date, 3 isoforms of nNOS have been identified; α , β and γ nNOS (Brenman et al. 1997; Eliasson et al. 1997). It is unclear whether either immunohistochemistry or NADPH-diaphorase activity is able to distinguish between the nNOS isoforms.

Estimates of the proportion of myenteric neurons that contain nNOS within the adult small intestine appear to vary greatly depending on species, strain, neuronal marker used and method of NOS detection used, as illustrated in table 10. Enteric neurons that utilise nitric oxide as a neurotransmitter have been studied in the adult intestine of several mammalian species (see table 10) as well as the embryonic (Balaskas et al. 1995b) and newly hatched (Balaskas et al. 1995a) avian intestine. However, postnatal development of the nitric oxide synthesising subpopulation of enteric neurons has not previously been described in mammals. Work presented earlier in this thesis (section 3.3) revealed changes in neuronal numbers during the first month of postnatal life, mainly increases in neuronal numbers between 7 and 14 days and between 21 and 28 days. A decrease in neuronal numbers was observed between 14 and 21 days. The aim of this study was to investigate whether there are postnatal changes in number and size of nitric oxide synthesising myenteric neurons and whether changes in this subpopulation contribute to the changes observed in the total population of myenteric neurons. The materials and methods for this investigation are presented in section 2.5.

Table 10 **Summary of reported estimates of the proportion of myenteric neurons that synthesis nitric oxide in the adult small intestine of several species.**

Species and Strain (where given)	Neuronal Marker	Marker for synthesis of NO	Approximate Percentage of total neurons	Reference
Mouse	Thionin stain	NADPH-d Histochemistry	33%	Grozdonovic et al. 1992
Guinea pig	NCB antisera	NADPH-d Histochemistry	19%	Furness et al. 1994
Wistar rats	NADH histochemistry	NADPH-d Histochemistry	27%	Cracco et al. 1994
Sprague Dawley rats	PGP 9.5 IHC	NADPH-d Histochemistry	27%	Belai et al. 1992
Rat (no strain info)	NADH histochemistry	NADPH-d Histochemistry	22%	Wilhelm et al. 1998
Guinea pig (no strain info)	NADH histochemistry	NADPH-d Histochemistry	19%	Wilhelm et al. 1998
Sprague Dawley rats	PGP 9.5 IHC	NADPH-d histochemistry	11%	Johnson et al. 1998
Sprague Dawley rats	NADH histochemistry	NADPH-d Histochemistry	22%	Johnson et al. 1998
BALB/c mice	PGP 9.5 IHC	NOS IHC	32%	Young et al.1998
Sprague Dawley rats	Cuprolinic blue	NOS IHC	28%	Jarvinen et al. 1999

Abbreviations

NCB	Neuron cell body
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PGP 9.5	Protein gene product 9.5
IHC	Immunohistochemistry
NOS	Nitric oxide synthase

5.3 Results

5.3.1 Effects of fixation

Attempts were made to double stain wholemount preparations of ileum for PGP 9.5 immunohistochemistry and NADPH diaphorase histochemistry in order to determine the proportion of myenteric neurons that can synthesise nitric oxide. NADPH diaphorase visualisation required paraformaldehyde fixation and a reaction product was undetectable when the tissue had been processed under the optimum conditions for PGP 9.5 immunohistochemistry (Zamboni's fixative, following treatment with an acetic acid rinse, see section 2.3). Although for the most part, this double labelling was successful (see figure 5a), as previously discussed (see section 3.3.3) visualisation of PGP 9.5 immunopositive myenteric neurons using paraformaldehyde fixation led to the rare presence of apparently unstained myenteric neurons. In order to obtain a more accurate assessment of neuronal density, pieces of tissue adjacent to those removed for NADPH diaphorase histochemistry were fixed and processed for PGP 9.5 as described in section 2.3. The PGP 9.5 immunostained preparations of ileum are discussed in more detail in chapter 3.

5.3.2 General appearance of NADPH diaphorase containing cells

In wholemount preparations of ileum of all ages, a subpopulation of myenteric neurons was found to contain NADPH diaphorase. It is unknown whether enteric glial cells synthesise nitric oxide and although only cells with a neuronal morphology were found to be NADPH diaphorase positive in the present study, the possibility that enteric glial cells contain NADPH diaphorase can not be ruled out.

During postnatal development, the morphology of nitric oxide synthesising neurons altered little, however at the youngest ages (7 and 14 days) the intensity of the histochemical reaction product exhibited variation (as seen in the double labelled preparations in figure 5a), possibly reflecting variation in the levels of NO synthesising enzyme at this time. NADPH diaphorase positive fibres were extensive and varicose in appearance throughout development.

5.3.3 Numbers of nitric oxide synthesising neurons

The numbers of NADPH diaphorase positive neurons were counted as described in section 2.5 and an absolute density (NADPH diaphorase positive neurons per cm² small intestine) calculated. In order to allow for the dilution effect that occurs as a result of small intestine growth, the absolute density was multiplied by a growth correction factor to give “corrected values”. The corrected values were then expressed relative to 7 day absolute density,, as discussed previously in section 2.3.5. These values for each age group are shown in table 11.

At the youngest age (7 days), the mean absolute density after correction for tissue stretch was 22013 +/- 509 NADPH diaphorase positive neurons per cm² of ileum. By 14 days, the mean absolute density was found to be 17797 +/- 688 neurons per cm² of ileum, giving a relative density of 1.56 +/- 0.06. The increase between 7 and 14 days was found to be highly significant (P<0.001, one way Anova), as shown in figure 5b. There was no significant change in the relative density of nitrergic neurons between 14 and 28 days. There was however a significant increase between 28 days and adulthood, suggesting that the full compliment of this population is not achieved until after the end of the first month of postnatal life.

Figure 5a Visualisation of nitric oxide synthesising myenteric neurons in double labelled preparations during postnatal development of the rat ileum.

Some masking of PGP 9.5 immunoreactivity occurred and double stained neurons exhibited blue cytoplasm and a green nucleus. The scale bar represents 50µm in A and B and 100µm in C-H. Asterisks indicate the probable location of cells which are PGP 9.5 immunonegative. Arrows indicate nitric oxide synthesising neurons. NADPH diaphorase activity in varicose fibres was apparent throughout development. As previously discussed,

- A A 7 day myenteric ganglion visualised by PGP 9.5 immunofluorescence. This image highlights the difficulty in counting neurons in preparations fixed with paraformaldehyde.
- B NADPH diaphorase positive neurons within the ganglion shown in A.
- C A 14 day myenteric ganglion visualised by PGP 9.5 immunofluorescence.
- D NADPH diaphorase positive neurons within the 14 day ganglion shown in C. Note the variation of staining intensity, suggesting variation in NO synthesising enzyme levels
- E A 21 day myenteric ganglion visualised by PGP 9.5 immunofluorescence.
- F NADPH diaphorase positive neurons within the 21 day ganglion shown in E.
- G A 6 month myenteric ganglion visualised by PGP 9.5 immunofluorescence. Note the PGP 9.5 negative cells with apparent neuronal morphology. These cells were found to represent 1%-3% of cells in paraformaldehyde fixed wholemount preparations. However, these cells were not apparent in preparations treated with acid rinse and Zamboni's fixative.
- H NADPH diaphorase positive neurons within the adult ganglion shown in G.

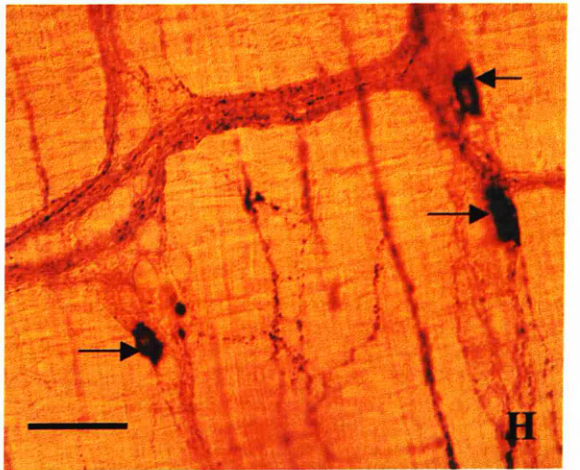
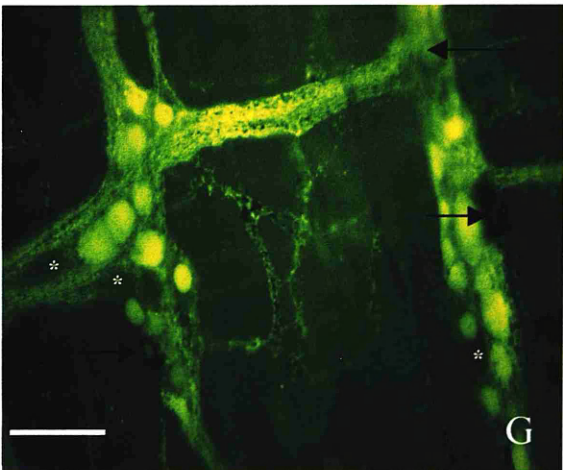
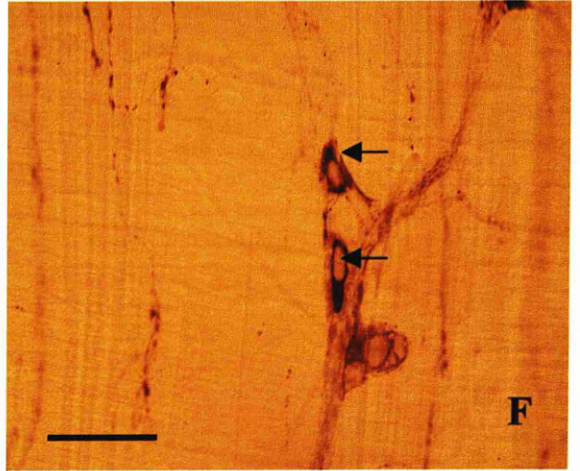
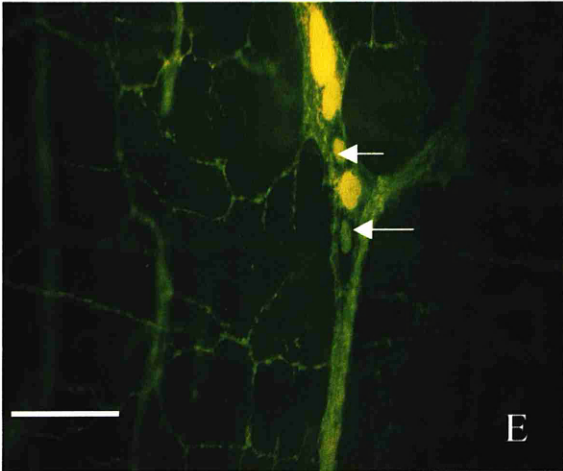
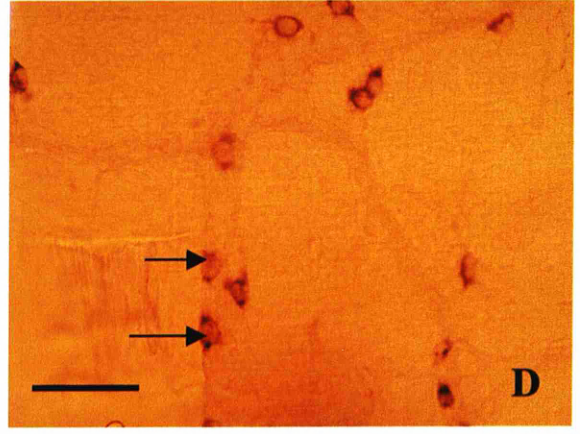
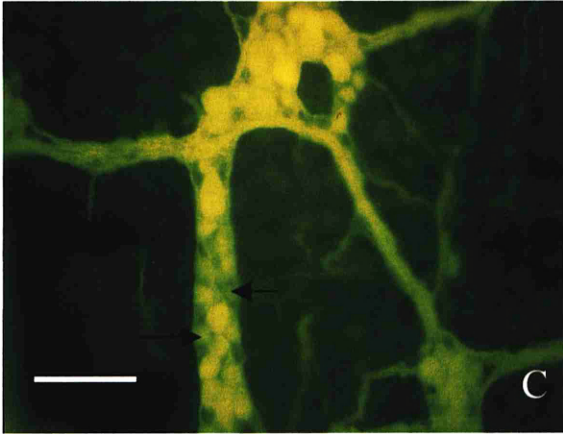
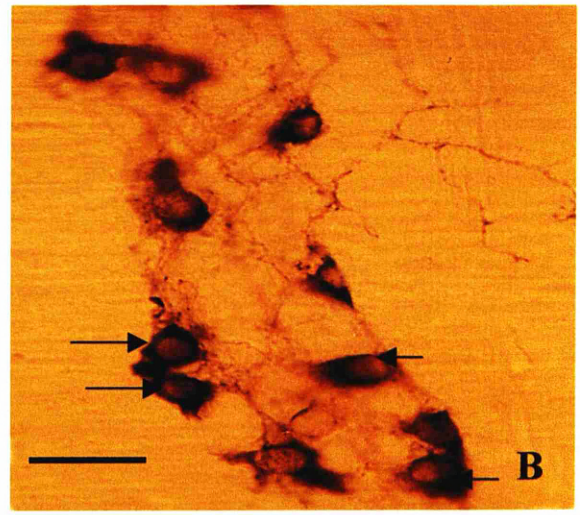
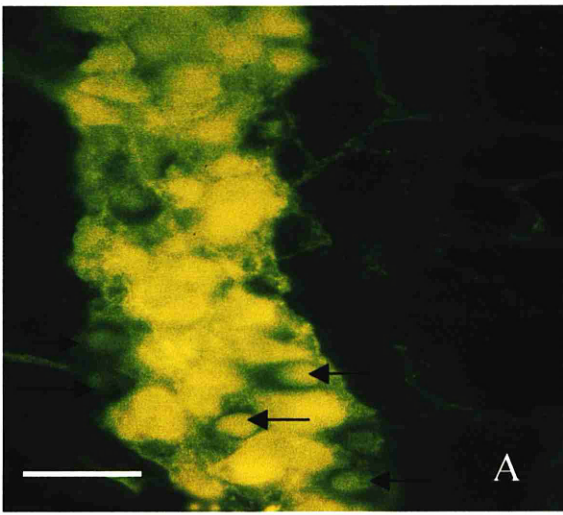


Table 11 **Changes in the numbers of nitric oxide synthesising myenteric neurons during postnatal development in the rat ileum.**

Age	Absolute density (+ve cells per cm²)	Growth Correction Factor	Corrected values	Ratio of corrected values to P7 values	Corrected total neuronal values	Ratio of corrected neuron values to P7 values
7 days	22013 +/-509	1.00	22013 +/-509	1.00 +/- 0.02	57575 +/- 900	1.00 +/- 0.02
14 days	17797 +/- 688	1.93	34348 +/- 1329	1.56 +/- 0.05	70882 +/- 424	1.23 +/- 0.01
21 days	9822 +/- 688	3.26	32030 +/- 910	1.45 +/- 0.04	54578 +/- 238	0.95 +/- 0.01
28 days	5946 +/- 218	5.34	31752 +/- 1193	1.44 +/- 0.06	107873 +/- 630	1.87 +/- 0.06
6 month	3223 +/- 181	10.50	36992 +/- 1905	1.68 +/- 0.10	139757 +/- 330	2.43 +/- 0.06

The chart summarises the mean number of NADPH diaphorase containing cells neurons per cm² small intestine (absolute density), following correction for general growth (Corrected values; absolute density multiplied by growth correction factor) and in relation to the corrected density at 7 days (corrected values divided by P7 absolute density). Corrected and relative values for total myenteric neurons are also given for comparison.

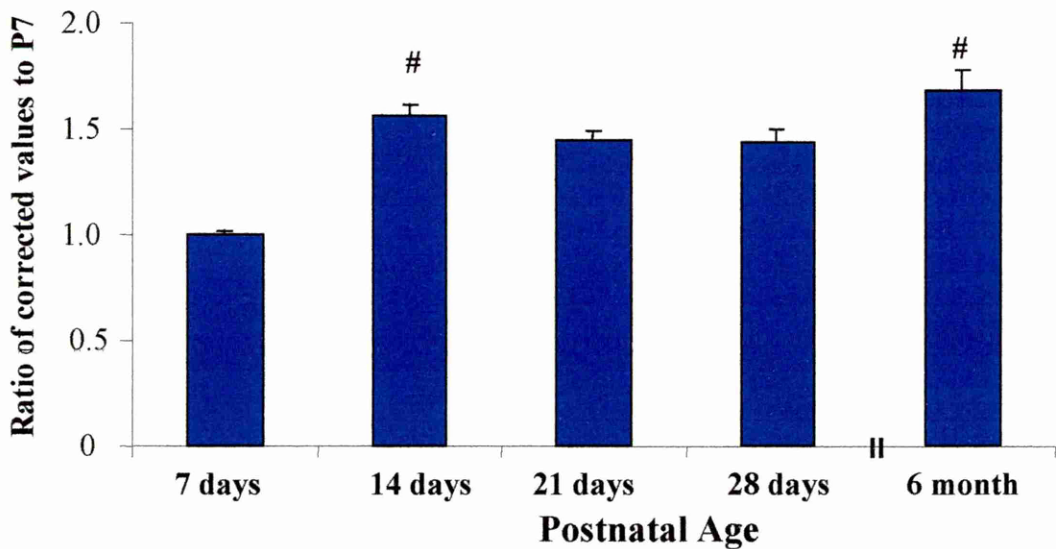
5.3.4 Proportion of myenteric neurons that contain NADPH diaphorase

The proportion of myenteric neurons that synthesise nitric oxide was calculated using the mean corrected numbers of myenteric neurons and mean corrected numbers of NADPH diaphorase positive cells at each age and the value obtained therefore is only an estimate. At 7 days, approximately 38% of myenteric neurons expressed NADPH diaphorase activity and by 14 days this figure had increased to 48%. The proportion of myenteric neurons containing this enzyme increased further, to 59% by 21 days. Between 21 and 28 days a significant decrease in the proportion of myenteric neurons containing NADPH diaphorase (from 53% to 29%) was observed. There was no significant change between 28 days and adulthood (26%), as illustrated by figure 5c.

5.3.5 Sizes of neurons containing NADPH diaphorase activity

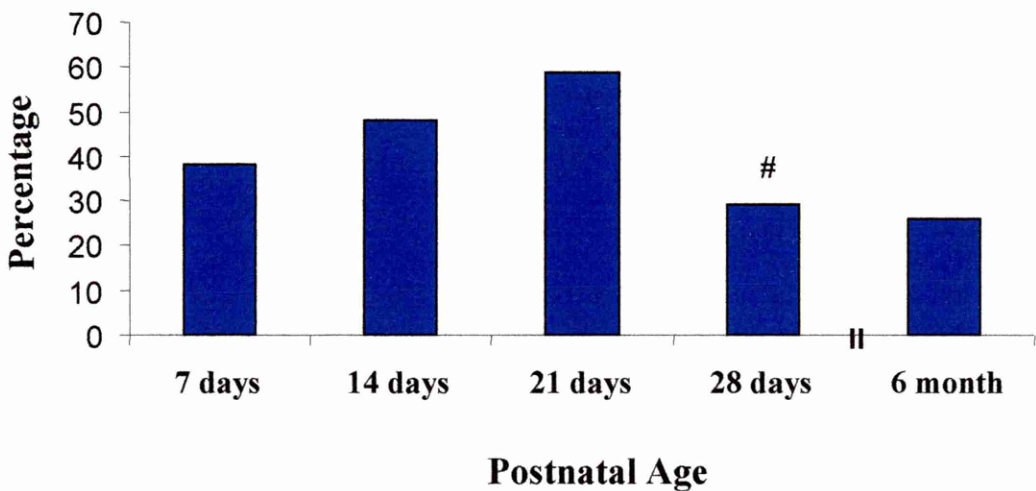
As a measure of neuronal size, somal area, maximum cell diameter, maximum cell length and maximum nuclear diameter was recorded for 150 neurons in each age group. Mean somal area was $139.65 \pm 3.56 \mu\text{m}^2$ at 7 days, increasing significantly by 14 days to $171.99 \pm 4.28 \mu\text{m}^2$. Mean somal area also increased significantly between 14 and 21 days, between 21 and 28 days, and between 28 days and adulthood, reaching $402.85 \pm 12.66 \mu\text{m}^2$ in 6 month animals. The increase in area appeared mainly to be due to an increase in cell body length. Significant increase in cell diameter occurred only between 14 and 21 days and between 28 days and adulthood. At 7 days, mean nuclear diameter was $9.57 \pm 0.15 \mu\text{m}$; there was no significant change in mean nuclear diameter until the end of the postnatal month when a significant increase from 10.78 ± 0.54 to $13.16 \pm 0.16 \mu\text{m}$ was observed.

Figure 5b Changes in the relative numbers of nitric oxide synthesising neurons in the developing rat ileum.



The mean relative values of NADPH-diaphorase positive neurons are shown per cm² ileum for each age group. +/- standard error. Hatched values (#) indicate significant difference from the previous value (p<0.001, one way Anova).

Figure 5c Changes in the proportion of myenteric neurons that synthesise nitric oxide during postnatal development



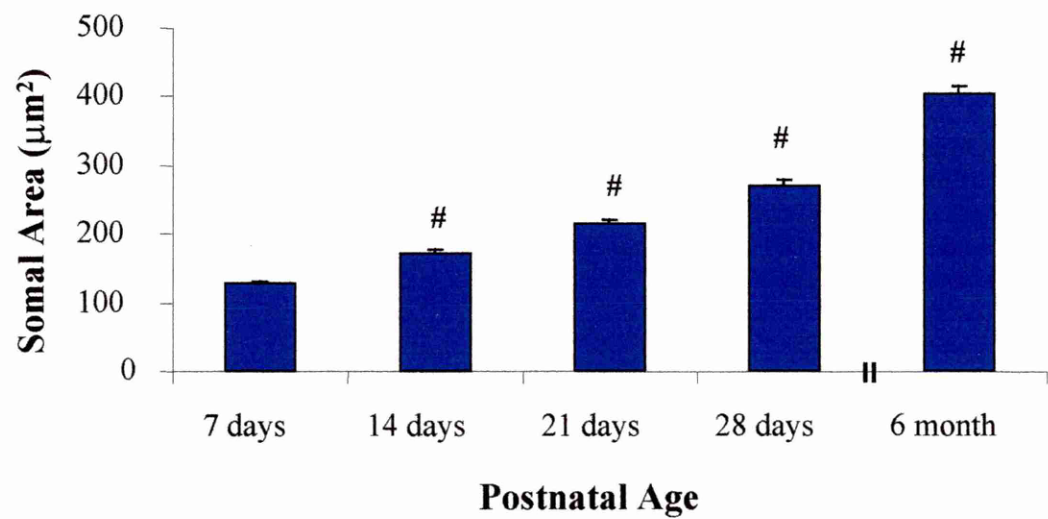
An estimate of the proportion of myenteric neurons (as determined by PGP 9.5 immunohistochemistry) containing NADPH diaphorase was calculated at each age. Hatched values (#) indicate significant difference from the previous value (p<0.001, one way Anova).

Table 12 Changes in the size of myenteric neurons containing NADPH diaphorase activity during postnatal development

AGE	Cell Body Area μm^2	Max Cell Diameter μm	Max Cell Length μm	Max Nucleus Diameter μm
7 days	128.53 +/- 3.56	11.44 +/- 0.24	17.65 +/- 0.26	9.57 +/- 0.15
14 days	171.99 +/- 4.26	13.09 +/- 0.27	21.00 +/- 0.30	10.82 +/- 0.13
21 days	215.17 +/- 5.59	15.31 +/- 0.27	23.19 +/- 0.40	11.02 +/- 0.12
28 days	269.02 +/- 10.24	15.73 +/- 0.36	28.50 +/- 0.64	10.78 +/- 0.54
6 month	402.85 +/- 12.66	19.39 +/- 0.45	33.07 +/- 0.67	13.16 +/- 0.16

The size of 150 myenteric neurons detected by NADPH diaphorase histochemistry was measured at each age and the mean +/- standard error is shown for each category.

Figure 5d Changes in mean somal area of myenteric neurons containing NADPH diaphorase activity during postnatal development



The mean somal area of 150 myenteric neurons detected by NADPH diaphorase histochemistry was measured at each age. The mean +/- standard error is shown for each age group. Hatched values (#) indicate significant difference from the previous value ($p<0.001$, one way Anova).

5.4 Discussion

The aim of the work presented in this chapter was to evaluate the postnatal development of nitrergic myenteric neurons using wholemount preparations of ileum processed for NADPH diaphorase histochemistry. Previous study (see section 3.5) revealed dramatic changes in the total number of myenteric neurons during the first month of postnatal life, notably an increase in neuronal numbers between 7 and 14 days, followed by a decrease between 14 and 21 days and an increase between 21 and 28 days, with a stable pattern apparently being established by 35 days. Results from the present study suggest that the observed increase in total neuronal numbers between 7 and 14 days could be due in part to an increase in the number of nitric oxide synthesising myenteric neurons (figures 5b and 5c). The relative numbers of the nitric oxide synthesising population remained relatively constant between 14 and 28 days, which is consistent with observations of the murine myenteric plexus (Young and Ciampoli 1998).

Between 14 and 21 days a decrease in total neuronal numbers was observed, however a decrease in the numbers of nitric oxide synthesising neurons was not observed during this time, therefore it is unlikely that there is a loss of neurons from this population and there may be a loss of neurons with other phenotypes. There is evidence to suggest that neurons containing NADPH diaphorase activity are resistant to excitotoxicity, as discussed previously (see section 1.5.2) and that this population of enteric neurons may be selectively spared in disease states (such as Alzheimer's and Huntingdon's diseases; Dawson et al. 1991) and during ageing (Santer 1994).

There was no change in the relative density of nitric oxide synthesising neurons between 21 and 28 days while the total number of myenteric neurons increased. This implies the addition of new neurons with other neurochemical phenotypes.

An increase in the numbers of nitric oxide synthesising neurons was observed between 28 days and adulthood, suggesting that the full compliment of this phenotype is not complete until after the end of the first postnatal month of life. This increase suggests either that nitrergic neurons are being “born” and differentiate and so effectively added to myenteric ganglia after the intestine has become relatively mature, or that a population of differentiated myenteric neurons acquire a nitrergic phenotype late during postnatal development either as an additional phenotypic characteristic, or as a result of a switch in phenotype. Postnatal withdrawal from the cell division cycle and differentiation of neurons containing VIP has been reported in the murine enteric nervous system during early postnatal life (Pham et al. 1991). In respect of how the additional neurons arise, it is unclear how they would be integrated into existing neuronal circuits and further work is needed to confirm both how the increase in neuronal numbers described in the present study is generated and to establish the location of the “new” neurons recorded in this study.

The observed increase in the nitric oxide synthesising population of myenteric neurons implies a possible increase in NANC inhibitory activity between 7 and 14 days and between 28 days and adulthood. Possible functions of such an increase at these times are unknown, however nitric oxide may have different or additional roles in the developing enteric plexuses, such as those observed earlier in development and from *in vitro* studies. For example, there is some evidence to suggest that nitric oxide may influence the phenotype of late differentiating neurons in the fetal murine enteric nervous system (Branchek and Gershon 1989), and that nitric oxide mediates NGF stimulated differentiation of PC12 cells (Peunova and Enikolopov 1995).

Previous studies on the quantitation of nitric oxide synthesising enteric neurons have focused on the adult small intestine and there is considerable variation between estimates of density, as illustrated by table 10. This variation could be due to a number of factors including; fixation (see section 3.3), method of visualisation, the method of area determination (allowing for the relaxation state and stretch during tissue preparation), as well as inter-strain differences. In the current investigation, the mean density of NO synthesising neurons in the adult was 3223 ± 181 per cm^2 ileum, corresponding to approximately 24% of total myenteric neurons. This figure is similar to that reported in previous studies on the adult rat ileum using PGP 9.5 as a neuronal marker (Belai et al. 1992b).

Measurements of somal size were compared during development in an attempt to assess maturity relative to the adult. The results show that mean somal area exhibited a series of significant increases during postnatal development, until there had been almost a 3 fold increase between 7 days ($139.65\mu\text{m}^2 \pm 3.56\mu\text{m}^2$) and adulthood ($402.85\mu\text{m}^2 \pm 12.66\mu\text{m}^2$). Maximum somal length increased significantly between all of the ages examined, however the same pattern was not observed with maximum somal diameter.

There was no significant increase in nuclear size or somal diameter during the first month of postnatal life, however there was a significant increase in both measures between 28 days and adulthood, suggesting that although relatively mature (with regard to size), the adult level of maturity may not be achieved until after the end of the first postnatal month. Possible stimuli for the observed size and density changes are unknown, although many concomitant changes occur within other gut layers.

Stimuli for postnatal development of myenteric neurons could include the action of nutrients obtained from the diet; either directly after being absorbed through the gut, or indirectly by acting on other organs or parts of the nervous system which in turn influence myenteric neurons. The presence of a number of neurotrophic factors has also been shown in the gastrointestinal tract and several of these have shown to influence postnatal myenteric neurons *in vitro* (see sections 1.7 and 6.1).

In summary, this study has shown postnatal changes in the density and size of nitric oxide synthesising neurons during postnatal development. The results suggest increases in density during the suckling period (between 7 and 14 days) and following the end of the first month of postnatal life. Possible signals for these increases are known, although effects directly related to diet change appear unlikely, as numbers of NADPH diaphorase containing neurons remained constant over the weaning period. The implications of increased nitrergic transmission during postnatal development are also unknown, however roles may include stimulating the differentiation of later developing neuronal phenotypes and therefore nitric oxide may not just be acting as a NANC neurotransmitter. The decrease observed in neuronal numbers was not observed in the population of nitric oxide synthesising neurons, suggesting that any neuronal loss during this time is unlikely to occur in neurons which synthesise nitric oxide.

CHAPTER 6

IN VITRO ACTIONS OF NEUROTROPHIC FACTORS ON MYENTERIC NEURONS

6.1 Abstract

GDNF has been shown to be essential for the normal embryonic development of the enteric nervous system. The aim of this investigation was to study the effects of GDNF on postnatal myenteric neurons in dissociated tissue culture. The results show that GDNF has neurotrophic actions, stimulating a dose-dependent increase in mean neurite length. Although overall cell numbers were not influenced by the neurotrophic factor, the proportion of neurons increased after treatment with 1ng/ml and 10ng/ml GDNF. Potential effects on somal area were also investigated and compared with other factors known to have neurotrophic effects on myenteric neurons in this system (CNTF and NT-3). As with CNTF and NT-3, GDNF had no effect on somal area at any concentration tested, suggesting that this feature of neuronal development may not be influenced by these factors. The results, together with reports that GDNF is present in the postnatal and adult intestine, suggest that this trophic factor may be important in the postnatal development and maintenance of the enteric nervous system.

6.2 Introduction

The work presented in previous chapters together with previous reports (Faussone-Pellegrini et al. 1996; Faussone-Pellegrini et al. 1999; Matini et al. 1997; Sakata and Setoyama 1997; Vannucchi et al. 1997; Vannucchi and Faussone-Pellegrini 1996) have shown the first month of postnatal life to be a time of substantial change in the mammalian intestine and enteric nervous system. Possible stimuli for the observed changes in neuronal numbers are unknown, however evidence suggests the involvement of factors which promote neuronal survival and/or differentiation, the so-called neurotrophic factors.

Neurotrophic factors have shown to be critical in embryonic neuronal development (Barbacid 1995; Davies 1996; Oppenheim 1989; Oppenheim 1996; Saarma and Sariola 1999) and recent evidence suggests that they are also involved in later development and maintenance of the nervous system. Several factors are known to be involved in the embryonic development of the enteric nervous system (see sections 1.6 and 1.7) and the lack of such factors may lead to severe or fatal effects, such as those observed in GDNF or c-Ret deficient mutant mice (discussed later).

Factors that regulate and maintain the postnatal and mature enteric nervous system are less well understood, although the presence of several neurotrophic factors has been detected in the postnatal and adult intestine (see Saffrey and Burnstock 1994 and table 7). Enteric ganglia undergo considerable change in shape during peristalsis and this is achieved by changes in shape of the enteric ganglion cells (Gabella 1990), and as a consequence enteric neurons may require a unique level (or type) of neurotrophic support.

In vitro studies have proved useful in assessing the role of potential neurotrophic factors during postnatal development. Postnatal myenteric neurons have been shown to respond to a variety of neurotrophic factors *in vitro*, a summary is shown in table 13. One such factor is the neurotrophin NT-3. Expression of NT-3 continues into postnatal life and myenteric neurons have been shown to express the NT-3 high affinity receptor (Trk C) throughout life (Esteban et al. 1998; Sternini et al. 1996). NT-3 has been shown to stimulate an increase in the number of myenteric neurons (Saffrey et al. 1996) and neurite outgrowth from myenteric neurons grown in cultures of dissociated myenteric plexus (Saffrey et al. 1997), however *in vivo* roles of NT-3 in the postnatal enteric nervous system are as yet unknown. Studies of mutant mice overexpressing NT-3 have shown that these animals exhibit increased numbers and size of myenteric neurons (Pham et al. 1996), however disruption of the high affinity neurotrophin (Trk) family of receptors in mutant mice has not revealed specific intestinal abnormalities (Pham et al. 1996), possibly indicating that the functions of this receptor are taken over by other receptors or that other molecules are able to compensate for the loss.

Ciliary neurotrophic factor (CNTF) is another factor that has been found to stimulate an increase in neurite outgrowth by myenteric neurons, both in rats (Silva et al. 1997) and guinea pigs (Grider et al. 1997a). The effect of CNTF is additive to that of NT-3 when the two are added in combination (Grider et al. 1997a), although the two factors have also been shown to have slightly different effects (Saffrey et al. 1997). CNTF has been shown to stimulate an increase in the number of secondary neurites extended by myenteric neurons, a so-called sprouting effect that may be involved in the repair and maintenance of enteric neurons (Saffrey et al. 1997). The use of blocking antibodies to CNTF has shown a dose dependent inhibition of neurite outgrowth, implying that CNTF is endogenous to myenteric ganglia (Grider et al. 1997a).

The α subunit of the tripartite CNTF receptor is expressed in the fetal intestine (Ip et al. 1991; Rothman et al. 1994) and mutant mice that lack this subunit die shortly after birth and appear to lack enteric motoneurons (see Gershon 1997).

Glial cell line-derived neurotrophic factor (GDNF) has been shown to be critical for the survival and differentiation of most of the enteric nervous system during embryogenesis (see section 1.7). Mice carrying null mutations of the GDNF gene were found to lack an enteric nervous system distal to the stomach (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996). Postnatal effects of GDNF on the enteric nervous system cannot to be characterised using knock-out animals, as the mice die shortly after birth from kidney defects (Schuchardt et al. 1994). These features are also exhibited in mutant mice with disruptions to the GFR α and cRet components of the GDNF receptor complex (Schuchardt et al. 1994), suggesting that the interaction between GDNF and the GFR α /cRet complex is critical for the early development of the enteric nervous system. There is evidence to suggest that GDNF can bind to GFR α and signal through an alternative (the Src-type) tyrosine kinase pathway (see Saarma and Arumae 1999; Saarma and Sariola 1999), although possible roles of this pathway are as yet uncertain.

Potential roles of GDNF in the postnatal enteric nervous system are as yet unknown, however the presence of GDNF has been demonstrated in the postnatal rat (Choi-Lundberg and Bohn 1995; Peters et al. 1998; Silva et al. 1999) and mouse (Golden et al. 1999) intestine and postnatal myenteric neurons have recently been found to be responsive to GDNF in culture (Schäfer and Mestres 1999).

Another GDNF family member (neurturin) may also have a role in enteric nervous system maintenance, as its presence has been detected in the adult mouse enteric nervous system (Golden et al. 1999) and myenteric abnormalities have been shown in neurturin deficient mice (Heuckeroth et al. 1999). Myenteric neurons of neurturin deficient mice are smaller and appear to extend fewer neurites than those of the wild type animals. Release of peptide neurotransmitters from myenteric neurons and smooth muscle contraction is also decreased in these animals (Heuckeroth et al. 1999).

The aim of this investigation was to examine possible effects of GDNF on postnatal myenteric neurons in an *in vitro* system. Possible effects on neuron and glial cell numbers, neurite length and somal area were examined. The potential influence of GDNF on somal area was compared to those of two other neurotrophic factors, CNTF and NT-3.

Table 13 Factors influencing the *in vitro* neuronal differentiation of postnatal myenteric neurons.

Factor	Species	Effect	Reference
BDNF	Guinea pig	Inhibition of neurite outgrowth	(Grider et al. 1997b)
bFGF	Guinea pig	Increase in neuronal numbers and neurite outgrowth	(Schäfer et al. 1995)
CNTF	Guinea pig	Increase in neurite outgrowth	(Grider et al. 1997a)
CNTF	Rat	Increase in numbers and length of neurites	(Silva et al. 1997) (Saffrey et al. 1997)
GDNF	Rat	Increase in neurite outgrowth	(Schafer and Mestres 1999)
IGF	Guinea pig	Increase in neurite outgrowth	(Mulholland et al. 1992)
NGF	Guinea pig	Increase in neurite outgrowth	(Mulholland et al. 1994)
NT-3	Rat	Increase in neuronal numbers and neurite outgrowth	(Saffrey et al. 1996) (Saffrey et al. 1997)

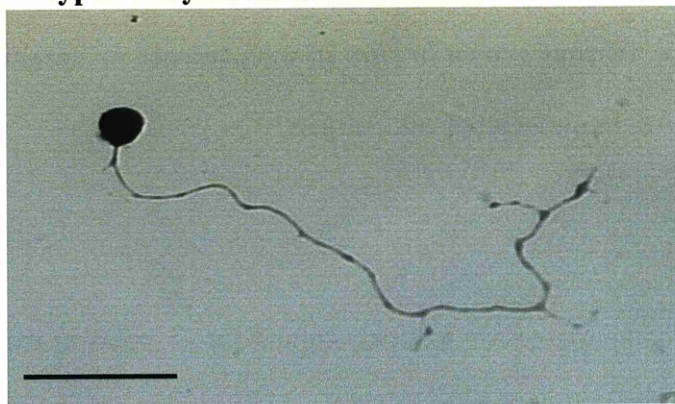
6.3 Results

6.3.1 General appearance of myenteric neurons in culture

The myenteric plexus of 7 day rat pups was grown in dissociated cell culture for 48 hours in the presence or absence of GDNF (as described in section 2.6), then processed for PGP 9.5 immunohistochemistry. Cells were seeded at a low density so that individual neurons could be examined. Neurons were easily distinguished from glial cells by both their morphology and by their high immunoreactivity for the neuronal marker PGP 9.5. Neurons had a rounded appearance whereas glial cells appeared flattened. Most, but not all neurons extended neurites and a variety of morphologies were observed, reflecting the diversity of myenteric neurons in the intact myenteric plexus (figure 6a and 6b).

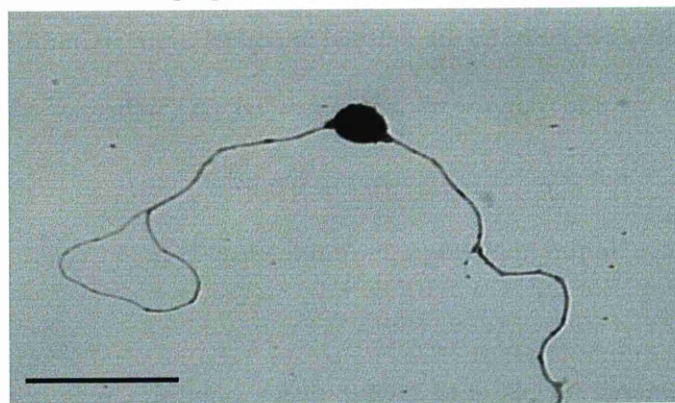
Neurons with neurites could be categorised into four groups by the number of primary processes extended; typical examples and descriptions are shown in figure 6a. It is unknown whether these neuronal types correlate directly with specific neurochemical phenotypes, although a straight forward correlation is unlikely as more than four different neurochemical phenotypes are present in the intact myenteric plexus (see Costa et al. 1987).

Figure 6a **Types of myenteric neurons observed in dissociated cell culture**



The types of myenteric neurons extending neurites were categorised into four groups on the basis of the number of neurites. Scale bars represent 50 μ m in each case.

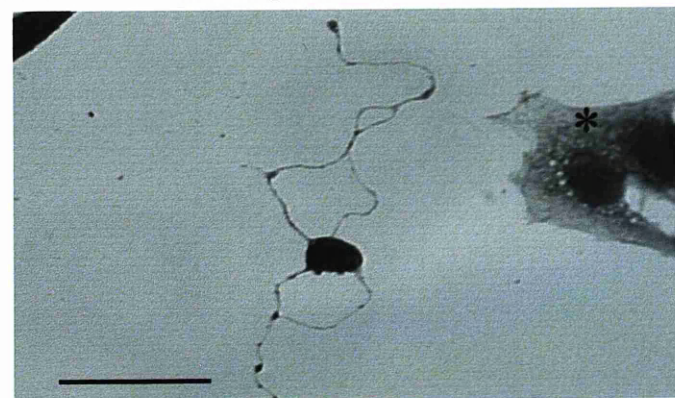
Type 1 neurons extend a single process which may or may not branch.



Type 2 neurons extend two neurites, often at opposite sides of the soma which may or may not branch.



Type 3 neurons extend several short processes, often evenly spaced around the soma.

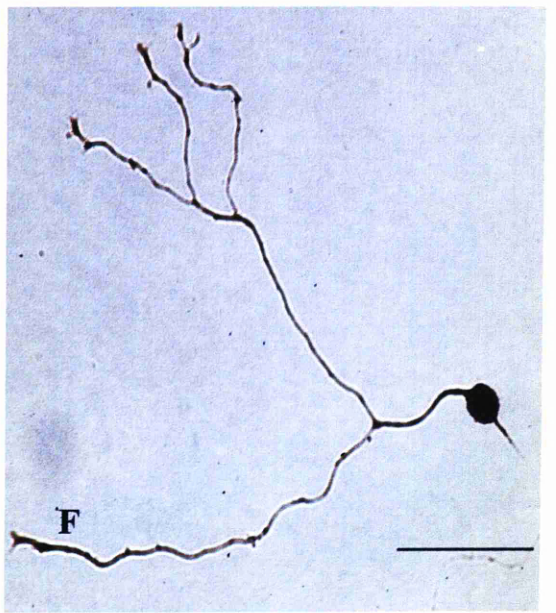
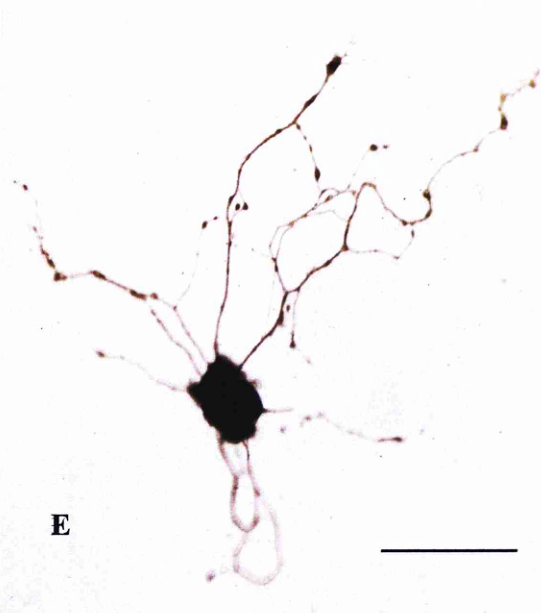
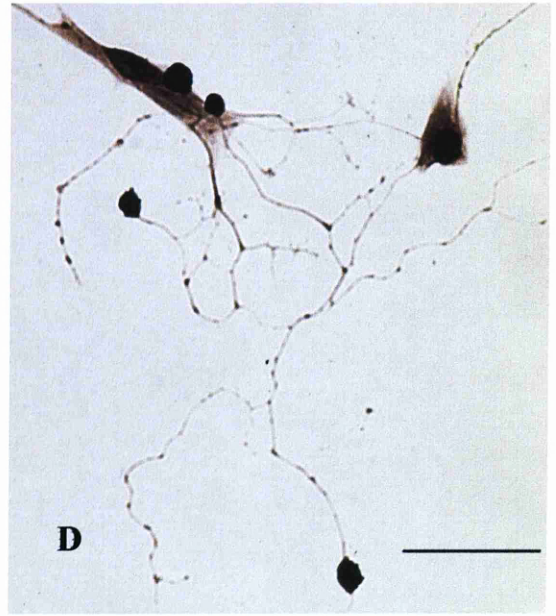
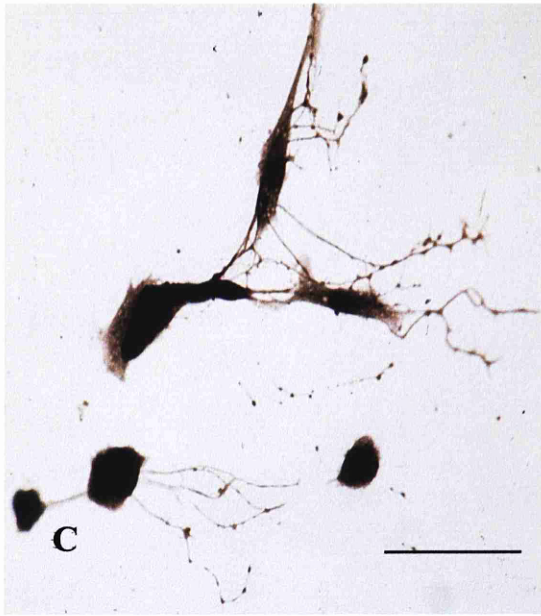
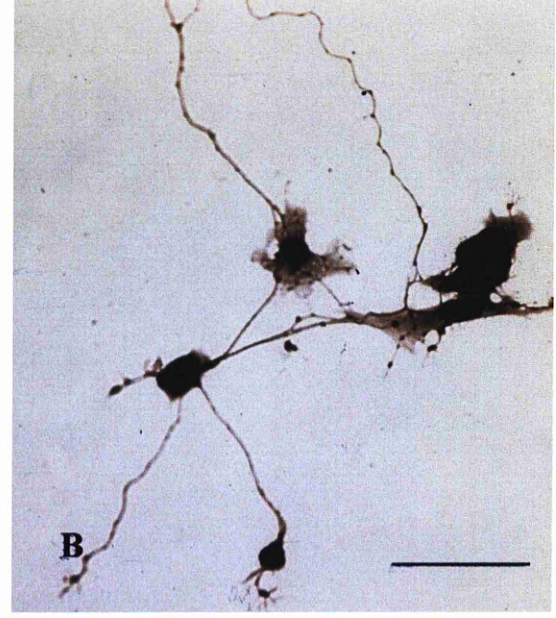


Type 4 neurons extend many long processes, often several times longer than the somal diameter.
* indicate glial cells.

**Figure 6b Myenteric neurons grown *in vitro* in the presence or absence of GDNF
for 48 hours in dissociated cell culture.**

Dissociated myenteric plexus was grown in tissue culture for 48 hours in the presence or absence of the neurotrophic factor GDNF and processed for PGP 9.5 immunohistochemistry. PGP 9.5 immunopositive neurons are stained black whereas glial cells appear brown. Neurons cultured in defined medium alone (Control conditions) exhibited only moderate neurite outgrowth was observed (A), whereas treatment with GDNF showed a concentration dependent increase in the mean length of neurite extension (B-D). Scale bar represents 100µm in photograph A and 50µm B-E.

- A Dissociated myenteric plexus was grown under control conditions in defined medium.
- B Dissociated myenteric plexus was grown in defined medium supplemented with 100pg/ml GDNF. Note the increased neurite extension.
- C Dissociated myenteric plexus was grown in defined medium supplemented with 1ng/ml GDNF.
- D Dissociated myenteric plexus was grown in defined medium supplemented with 10ng/ml GDNF. Note increased number and length of neurites.
- E A type 4 neuron grown in the presence of 10ng/ml GDNF. Note the number and length of neurites.
- F A type 1 neuron grown in the presence of 10ng/ml GDNF. Note the length of the neurites.



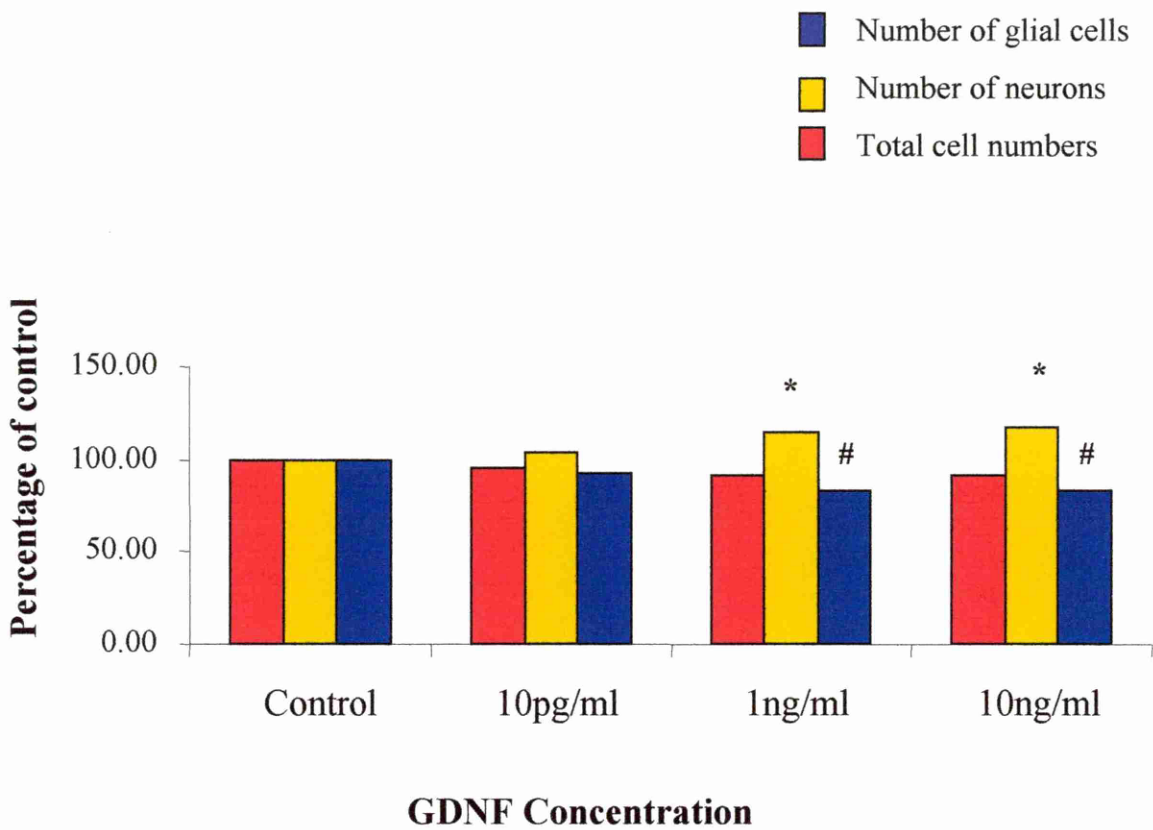
6.3.2 Neuron and glial cell numbers

Potential effects of GDNF on neuronal and glial cell numbers were examined by counting neurons and glial cells in a strip along the diameter of each coverslip, as described in section 2.6.2. A single field of view corresponded to 0.15mm^2 . A significant increase was observed in the number of myenteric neurons following treatment with either 1ng/ml or 10ng/ml GDNF when compared with control cultures ($p<0.05$, Anova).

A significant decrease in the number of glial cells was observed in cultures treated with either 1ng/ml or 10ng/ml GDNF and as a result there was no overall change in the total number of cells in cultures supplemented with GDNF compared with cultures grown in the absence of this factor (see figure 6c).

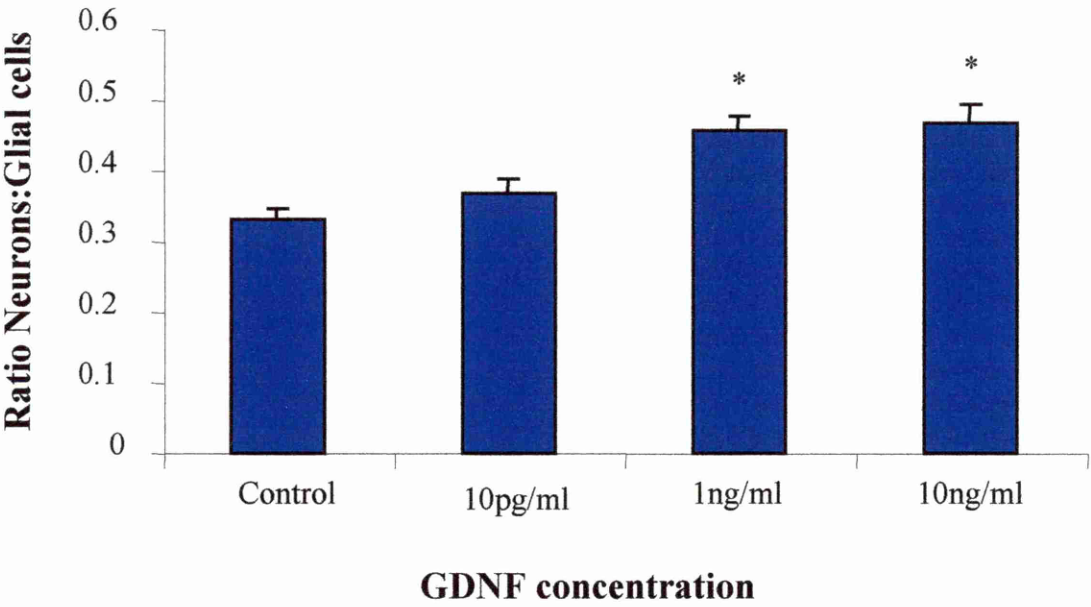
In control cultures, the mean ratio of neurons to glial cells was 0.33 (+/- 0.15), addition of 10pg/ml GDNF produced an increase in the ratio to 0.37 (+/- 0.19), however this was not significantly ($p<0.05$ significance level, two way Anova) different from the control. The ratio of neurons to glial cells increased to 0.46 (+/- 0.20) on addition of 1ng/ml GDNF, which was significantly different to the control ($p<0.05$ significance level, two way Anova). A further significant increase in the proportion of neurons to glial cells was observed at the highest concentration of GDNF (10ng/ml), 0.47 (+/- 0.26). There was no significant difference between the neuronal:glial cell ratios recorded after treatment with 1ng/ml and 10ng/ml GDNF. This concentration dependent increase in the proportion of neurons to glial cells is shown graphically in figure 6d.

Figure 6c Changes in the numbers of myenteric neurons and glial cells following treatment with GDNF



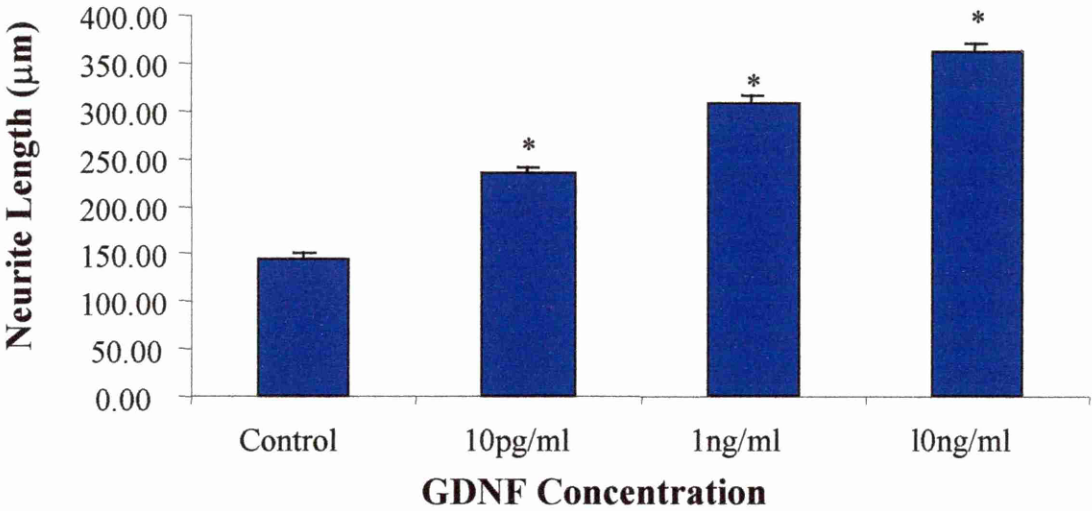
The mean number of neurons of glial cells was calculated and compared in each condition. A significant increase in the number of neurons was observed following treatment with either 1ng/ml or 10ng/ml GDNF (indicated by*, $p<0.05$, Anova), when compared with the control. A significant decrease in glial cell numbers was observed following treatment with either 1ng/ml or 10ng/ml GDNF (as indicated by #, $p<0.05$, Anova). There was no overall change in total cell numbers following treatment with GDNF.

Figure 6d **Changes in the ratio of neurons to glial cells *in vitro* following treatment with GDNF**



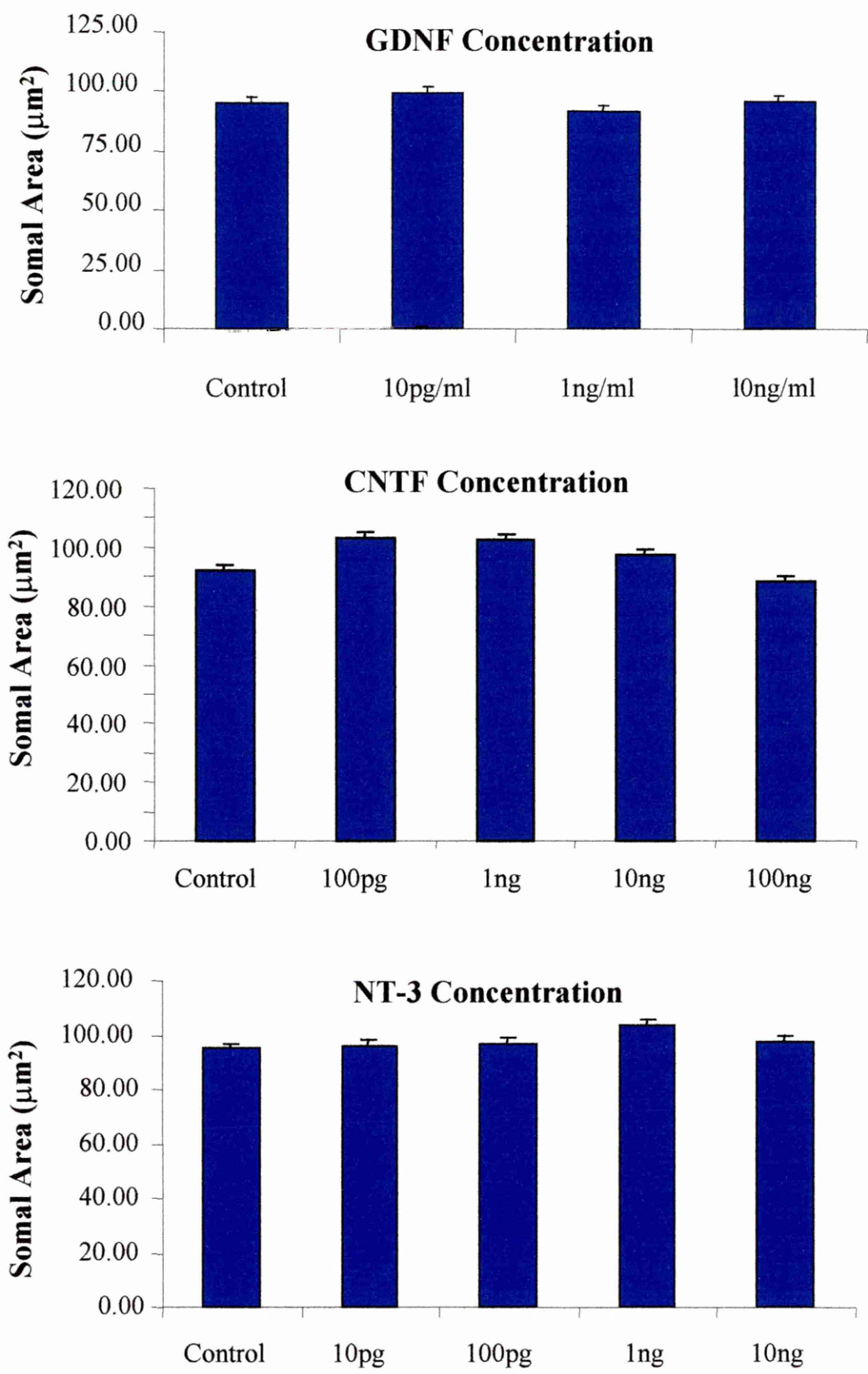
The number of neurons to glial cells were counted and the ratio of neurons to glial cells was calculated. The mean of three coverslips, repeated in triplicate is shown +/- SE for each concentration. * indicates significant difference from the control at the $p < 0.05$ level (two way Anova).

Figure 6e **Changes in mean neurite length *in vitro* following GDNF treatment**



Mean total neurite length was measured for 50 neurons per coverslip, three coverslips were counted for each condition and the experiment was repeated in triplicate. The mean is shown +/- SE. * indicates significant difference from the control at the $p < 0.05$ level (two way Anova).

Figure 6f Changes in somal area following neurotrophic factor treatment



The effect of different neurotrophic factors was examined on somal area. The area of 50 neurons were recorded for each coverslip, three coverslips were assessed in each condition and the experiment was repeated in triplicate. The mean are +/- standard error (SE) is shown below. The bar charts show that none of the factors at any of the concentrations tested had a statistically significant effect on somal area in this culture system ($p < 0.05$, two way Anova).

6.3.3 Neurite length

GDNF had a clear and consistent effect on neurite extension by cultured myenteric neurons. Neurons cultured in the presence of GDNF extended longer processes than those in sister cultures grown in the absence of trophic factor (figures 6b and 6e). In the present study however, specific effects on individual types of process-extending neurons were not quantified, however a shift from one type to another may occur as processes are extended.

Measurement of the total neurite length per neuron of 150 neurons per condition in each of three experiments showed that mean neurite length increased in the presence of GDNF. The mean neurite length for myenteric neurons cultured under control conditions was $144.95 \pm 5.95 \mu\text{m}$. Addition of 10pg/ml GDNF led to an increase in the mean neurite length ($234.81 \pm 6.82\mu\text{m}$). The mean neurite outgrowth increased significantly to $307 \pm 8.66\mu\text{m}$ in the presence of 1ng/ml GDNF ($p < 0.05$, two way Anova). Following treatment with 10ng/ml GDNF, mean neurite length was $360.15 \pm 9.52\mu\text{m}$ which was a significant increase compared to mean neurite length in the control ($p < 0.05$, two way Anova). There was no significant difference between the mean neurite length observed following treatment with either 1ng/ml or 10ng/ml GDNF.

6.3.4 Somal area

The area of 150 neurons in each treatment condition was measured and the mean calculated (figure 6f). Possible effects of GDNF on somal area were compared with those of other neurotrophic factors (CNTF and NT-3) known to stimulate neurite outgrowth from myenteric neurons *in vitro*. Figure 6f shows that none of the neurotrophic factors stimulated a significant increase in somal size, even at high concentrations (GDNF and NT-3 10ng/ml , CNTF – 100ng/ml). Although not quantified in the present study, neurons in contact with glial cells appeared larger than neurons not in contact with other cells.

6.4 Discussion

The results presented in previous chapters show that the postnatal myenteric plexus undergoes substantial change during the first month of life; changes in the density and distribution of myenteric neurons, as well as ultrastructural changes are observed. The stimuli for such changes are unknown, however the presence of several neurotrophic factors in the postnatal intestine and the responsiveness of myenteric neurons to some of them *in vitro*, suggests an involvement of these molecules. The aim of this investigation was to assess possible effects of one of these molecules, GDNF, on postnatal myenteric neurons in dissociated cell culture.

The results presented in this chapter show that GDNF has trophic actions in the postnatal enteric nervous system *in vitro*, as both the number of myenteric neurons and mean neurite length increased following treatment with this trophic factor. At concentrations of either 1ng/ml or 10ng/ml, GDNF produced an increase in neuronal numbers. A concomitant decrease in the number of glial cells was also observed after treatment with either 1ng/ml or 10ng/ml GDNF, and as a consequence there was no overall increase in the number of total cells following GDNF treatment. The results from the present study are in contrast to those reported by Schäfer et al. (1999) who found that concentrations of up to 100ng/ml did not influence the numbers of neurons in dissociated cultures from Wistar rat small intestine.

One explanation for the observed increase in the number of myenteric neurons is that GDNF is stimulating the differentiation of enteric precursors to a neuronal phenotype, rather than either the selective survival of differentiated neurons or the proliferation of precursors. The former explanation is more likely because total cell numbers did not change.

Undifferentiated precursors have been detected in the postnatal murine enteric nervous system, however it is unproven whether they are present in the postnatal rat myenteric plexus. GDNF does not appear to be acting as *general* survival factor in this system as there was a decrease in glial cell numbers at the highest concentrations tested. Further work is needed to establish the mechanisms leading to the observed increase in neuronal numbers and decrease in glial cell numbers in this system.

Treatment with GDNF also stimulated an increase in mean neurite outgrowth from cultured myenteric neurons, in a similar dose-dependent manner to that observed for CNTF (Grider et al. 1997a; Silva et al. 1997). The highest concentration tested (10ng/ml) stimulated an approximately 3 fold increase when compared with neurons grown in defined medium alone.

A variety of neuronal morphologies were observed during this investigation, however somal area appeared to be unaffected by treatment with a range of concentration of three neurotrophic factors to which myenteric neurons are known to be responsive (CNTF, GDNF and NT-3). It is possible that the neurotrophic factors tested may require the presence of other molecules (not present in the culture medium) in order to influence neuronal somal size. Neurons contacting glial cells appeared larger than isolated neurons (see figure 6e, and Schafer and Mestres 1999), however this effect was not quantified here. This observation suggests that other as yet undescribed, possibly glial cell derived and possibly cell surface molecules, influence somal area to generate the variety of neuronal sizes observed *in vivo* (see section 3.3.4).

In other culture systems, GDNF has been found to require TGF β to exert its full neurotrophic potential (Kriegstein et al. 1998). The cultures used in this present study were briefly exposed to FCS, which is a source of TGF β (Kriegstein et al. 1998).

The effects observed here, both on neuronal numbers and neurite outgrowth may thus have been due to the combination of these factors. The precise mechanism of GDNF action in this system is as yet unknown. GDNF may be acting directly on myenteric neurons or indirectly via glial cells that are also present in these cultures. However, since glial cell numbers were fewer in the treated cultures and enteric neurons have been shown to express the GDNF receptor cRet (Tsuzuki et al. 1995) it is more likely that the effects observed in this study were due to direct actions on myenteric neurons.

There is evidence to suggest that intestinal smooth muscle (Peters et al. 1998) is a source of GDNF in the intact postnatal intestine, suggesting that GDNF may act as a target-derived neurotrophic factor for some myenteric neurons. Enteric glial cells may also be a source of GDNF (Bar et al. 1997). Physiological roles of GDNF in the postnatal intestine remain to be elucidated, however GDNF protein has been detected along the length of the rat intestine throughout the postnatal period, with particularly high levels (0.76 pg/ml protein) of GDNF in the adult (Peters et al. 1998). The actual levels of GDNF that myenteric neurons are exposed to *in vivo* are not known, but the levels used here *in vitro* are likely to be higher than physiological levels.

In summary, this work has shown that GDNF has trophic actions on postnatal myenteric neurons in dissociated cell culture, stimulating increases in the proportion of neurons and mean neurite length. Overall cell numbers and somal area were not influenced by GDNF treatment. These results, together with the observation that GDNF is present in the postnatal and adult gut, suggest that GDNF may be important in the postnatal development and maintenance of the postnatal enteric nervous system.

CHAPTER 7

GENERAL DISCUSSION

Current knowledge of the structure, function and neurochemistry of the adult mammalian enteric nervous system is now extensive. This is mainly due to advances, during the last 30 – 40 years, in the techniques that are available to study this unique system. Study of the enteric nervous system in the adult intestine has revealed several characteristics of this system that distinguish enteric ganglia from other autonomic ganglia, while showing some similarities to the central nervous system, as revealed by techniques such as electron microscopy and immunohistochemistry. It is now firmly established that the enteric nervous system is a complex system and is essential for normal gastrointestinal functions (see Costa et al. 1991; Costa et al. 1987; Furness and Costa 1980).

The importance of the enteric nervous system is demonstrated in conditions where it is disrupted, such as Hirschsprung's disease where the enteric nervous system has failed to develop in varying lengths of the colon. At present there is no treatment for this disease and the aganglionic segment has to be surgically removed. Other clinical gastrointestinal conditions are associated with abnormalities of the enteric nervous system such as Crohn's disease (where abnormal levels of the neuropeptides VIP, substance P and somatostatin are observed, see Collins et al. 1997) and Chaga's disease where enteric ganglia have abnormal morphology (see Goyal and Hirano 1996). Abnormalities of the enteric nervous system have also been observed in the ageing mammalian intestine (see El-Shalhy et al. 1999; Feher 1992; Gabella 1989; Gomes et al. 1997) and in diseases that primarily affect other regions of the body, such as in Parkinson's disease and diabetes mellitus (see Goyal and Hirano 1996).

Therefore, it is important to understand how the enteric nervous system develops and how it is maintained during maturity. The early development of the enteric nervous system is becoming increasingly well understood, however little is known about its postnatal development. Recent studies suggest that although relatively well developed at birth, development of the mammalian enteric nervous system continues into the first postnatal month, a time when the intestine has become crucial for survival.

The aim of work undertaken here was to investigate potential changes in myenteric neuronal numbers during the first month of postnatal life and to study potential stimuli for such changes.

Immunohistochemistry for a neuronal marker, PGP 9.5 was used to study the total population of myenteric neurons and revealed a significant increase in neuronal numbers between 7 and 14 days. A second significant increase in neuronal numbers was shown between 21 and 28 days. Unexpectedly, a decrease in neuronal numbers was observed between 14 and 21 days. Establishment of the adult complement of myenteric neurons occurred after five postnatal weeks.

An ultrastructural study revealed myenteric ganglia to be relatively well developed at 7 days, having a dense neuropil and a variety of neuronal and glial cell profiles. The basal lamina, which is observed surrounding the majority of myenteric ganglia in the adult, was shown to be developmentally acquired, although a small proportion of myenteric ganglia in the adult ileum appeared to remain in direct contact with surrounding tissue as they had no visible basal lamina. Developmental changes were also observed in the smooth muscle layers surrounding myenteric ganglia. Between 7 and 21 days, the number of muscle cell profiles increased significantly and from 21 days onwards the thickness of the smooth muscle layers increased.

Both increases were recorded in the circular and the longitudinal muscle layers. Specialisations within the circular muscle layer became apparent from 21 days and widespread from 28 days.

Using NADPH diaphorase histochemistry, a significant increase in the number of nitric oxide synthesising neurons was shown between 7 and 14 days. The size of this subpopulation of myenteric neurons remained relatively constant until 28 days when a second significant increase was observed. The adult complement of nitrergic neurons appeared to be achieved after 28 days, as there was a further increase in the number of nitric oxide synthesising neurons between 28 days and 6 months.

Possible stimuli for the changes described above are unknown, however neurotrophic factors have been shown to be important in the embryonic development of the enteric nervous system and the presence of some of these factors has been described in the postnatal enteric nervous system and intestine (see Saffrey and Burnstock 1994). The actions of one such factor, GDNF, were examined on postnatal myenteric neurons *in vitro* and this factor stimulated a significant, dose-dependent increase in mean neurite length. At concentrations of 1ng/ml and 10ng/ml GDNF also stimulated a significant increase in the number of neurons and a concomitant decrease in the number of glial cells. Three trophic factors shown to stimulate neurite outgrowth (GDNF, NT-3 and CNTF) were found to have no effect on mean neuronal somal area.

A major finding of the work described in this thesis is the change in neuronal numbers during the first month of postnatal life. This period has been shown to be one of ongoing neurochemical differentiation in the murine enteric nervous system (Matini et al. 1997; Vannucchi et al. 1997; Vannucchi and Faussone-Pellegrini 1996).

The postnatal period is a time of great change for the rat pup and general growth was relatively rapid during this time, as indicated by the increase in body mass. During the third week of postnatal life, weaning usually occurs and the progression from a liquid high-fat low-carbohydrate diet of maternal milk to a solid high-carbohydrate diet has associated changes in gut architecture and biochemistry as previously discussed (and see Thompson and Keelan 1986).

The total number of myenteric neurons increased between 7 and 14 days and between 21 and 28 days, possibly suggesting that undifferentiated neural precursors are present and generate myenteric neurons in the postnatal rat enteric nervous system, as has been reported in the mouse (Pham et al. 1991). The neurochemical phenotype of those neurons added to the myenteric plexus between 7 and 14 days includes nitric oxide synthesising neurons, as discussed previously. VIP and PACAP immunoreactive neurons were found to undergo their terminal differentiation during the postnatal period in the murine small intestine (Matini et al. 1997) and if the rat enteric nervous system follows a similar developmental pattern as that of the mouse, then VIP and PACAP immunoreactive neurons may be expected to be among the “new” neurons observed in this investigation.

The increases in neuronal numbers between 7 and 14 days and between 21 and 28 days, imply that “new” neurons are being added to established ganglia, although how such cells are generated and integrated into the myenteric plexus remains to be characterised. Possible mechanisms could involve the differentiation of precursors that may or may not be capable of dividing. Birthdating techniques, such as the incorporation of either tritiated thymidine or BrdU would permit the detection of precursors undergoing DNA synthesis, as used previously to detect precursors in the murine enteric nervous system (Pham et al. 1991).

Immunolabelling, using antisera which recognise molecules only expressed by precursor cells, such as nestin could also be used to detect the presence of undifferentiated precursors (Chalazonitis et al. 1998). The presence of undifferentiated precursors has also been implied indirectly by the observation described here that GDNF can stimulate an increase in the number of myenteric neurons *in vitro* while the total number of cells in culture did not change.

The phenotype of the “new” neurons that are detected in the postnatal myenteric plexus remains to be firmly established. The work described here however indicates that nitrergic neurons are among neurons that are being added to myenteric ganglia between 7 and 14 days. The second increase in myenteric neuronal numbers occurs between 21 and 28 days. Although there is a second increase in the nitric oxide synthesising subpopulation of myenteric neurons, this occurs between 28 days and adulthood so it is unlikely that this subpopulation contribute to the increase in neuronal numbers observed between 21 and 28 days. A possible switch in neuronal phenotype or concomitant loss of another neuronal subpopulation after 28 days has already been discussed (see section 6.4)

A decrease in neuronal numbers, such as that observed between 14 and 21 days in the present study, would represent an effective loss of neurons from the myenteric plexus. Such a loss could result either from the death or the migration of myenteric neurons. In other regions of the nervous system a period of naturally occurring cell death by apoptosis follows establishment of connections between neurons and their target cells (see Burek 1996; Clarke 1990; Henderson 1996; Vaux and Korsmeyer 1999). This period appears to be one of reorganisation and elimination of excess connections following the innervation of targets, used to establish appropriate connections.

There are several targets for the different subpopulations of myenteric neurons, including other myenteric neurons, smooth muscle cells, submucosal neurons and the mucosal epithelium, making the myenteric plexus a complex meshwork of neuronal microcircuits and hence a difficult system in which to determine the explicit effects of target derived factors. However, prior to the observed decrease in relative neuronal density is a significant increase that potentially could be an overproduction of myenteric neurons in order to ensure appropriate connections can be made (as observed with spinal motoneurons, (see Oppenheim 1996).

The use of markers of apoptosis such as detection of fragmented DNA (for example by the Tunel and Fragel methods) has been used to study the possibility of naturally occurring cell death in other regions of the nervous system (for example, Goping et al. 1999; Whiteside et al. 1998; Whiteside and Munglani 1998). Electron microscopy has also been utilised to detect apoptotic neurons (for example, Goping et al. 1999). No evidence for apoptosis was detected in the present study, although the sample numbers were too low to be able to discount this possibility with certainty. Problems of studying developmental cell death in the enteric nervous system are caused by the variability and irregularity of enteric ganglia as well as the speed (as shown from other nervous system regions) with which dying neurons would be removed by neighbouring cells. Whether the decrease in number of myenteric neurons occurs within a specific neuronal lineage or phenotype is also unknown, although there appears to be no loss of nitric oxide synthesising neurons at this time. Neuronal losses may be associated with their location and projections relative to potential sources of survival factors. Studies of the different lineages and of neurochemical markers during postnatal life are necessary to further investigate this question.

An alternative explanation for a decrease in neuronal numbers in the myenteric plexus is that myenteric neurons are migrating to the submucous plexus. The finding that the submucous plexus consistently develops later than the myenteric plexus (Matini et al. 1997; Vannucchi et al. 1997; Vannucchi and Fausone-Pellegrini 1996), would support this suggestion. A concomitant increase in the number of submucosal neurons would be consistent with this suggestion and an investigation of possible postnatal changes in the numbers of submucosal neurons and the use of cell marking techniques would be required to confirm this possibility.

Estimates of neuronal numbers within the adult enteric nervous system exhibit a great deal of variation, according to the methods used, the species and strain examined, as well as the gut region studied. Such variation, even within species emphasises the need for standardisation of variables, such as tissue stretch or shrinkage. These problems are compounded when comparing neuronal numbers between animals at different ages as the intestine grows substantially, leading to possible detection of false decreases in neuronal density as neurons appear to be spread over a greater area. In the present study, in order to allow for intestinal growth, a relative number of myenteric neurons was calculated and compared. Total neuronal numbers (per cm² of ileum) were determined for tissue at each age and multiplied by a growth correction factor, resulting values were then expressed as a ratio to 7 day neuronal numbers. Calculation of a growth correction factor from small intestinal area is based on the assumption that the small intestine grows uniformly. While this is not an unreasonable assumption, it is not certain and could only be ascertained by further study, in which the growth of individual regions of the small intestine was determined.

Morphological studies performed here showed that, although the myenteric ganglia appeared well developed, they were incompletely covered by a basal lamina in young animals. Changes in the basal lamina and its constituent molecules (such as laminin) could influence growth of myenteric neurites into the surrounding smooth muscle. An incomplete basal lamina could also enable increased exchange of molecules between the ganglia and surrounding tissues. The observation that smooth muscle thickness increases at the same time that an increase in neuronal numbers is detected suggests a role of intestinal smooth muscle in the regulation of neuronal numbers, as suggested previously. This would suggest that the "new" neurons are motoneurons and they could be excitatory (e.g. cholinergic) or inhibitory (e.g. nitrenergic).

Direct studies to confirm a role for target-derived factors from intestinal smooth muscle in the development of the enteric nervous system are difficult to perform as grafting of additional muscle (as has been done for skeletal muscle in the study of motoneuron cell death, for example see Hollyday et al. 1977) is not easy to perform in the gut. Artificially induced hypertrophy of smooth muscle by stenosis (Gabella 1993) could be attempted in younger animals, but such methods are problematic. Indirect studies to investigate the possible production of trophic factors by smooth muscle could include the co-culture of smooth muscle with myenteric neurons. A recent study has shown that a protein extract from homogenised gut wall has neurotrophic effects on cultured rat myenteric neurons (Schäfer et al. 1998). GDNF is a candidate for (at least some) of this neurotrophic effect as GDNF is known to be present in the postnatal gut wall (Peters et al. 1998). The present results, also using a tissue culture system of isolated myenteric ganglia, indicate that GDNF may have trophic actions in the postnatal myenteric plexus and support the recent report by Schäfer (Schäfer and Mestres 1999). Although *in vitro* systems have been useful in identifying factors that elicit responses from postnatal myenteric neurons, responses to that factor *in vivo* may be different.

Although GDNF, CNTF and NT-3 have been shown to exert neurotrophic effects on myenteric neurons (see section 6.3), none of these factors were able to stimulate an increase in the size of myenteric neurons. This, and other aspects of neuronal growth and maintenance, may require molecules additional to neurotrophic factors. In another culture system, it has been shown that the presence of TGF β is needed for the full neurotrophic potential of GDNF to be seen (Kriegstein et al. 1998). The observation that myenteric neurons in contact with glial cells are often larger than those on the poly-l-lysine substrate could suggest that GDNF requires a cell surface or extracellular matrix molecule in order to exert its full neurotrophic potential upon myenteric neurons in this culture system. A major candidate for such a factor is laminin, which has been shown to be produced by enteric glial cells in culture (Bannerman et al. 1988).

The array of trophic factors observed in and around the postnatal enteric nervous system may have multiple and overlapping roles, as suggested by the lack of detectable deficit in the Trk-C knock out mice (Tessarollo et al. 1997; Wyatt et al. 1999) and the way that several unrelated factors elicit increases in neurite outgrowth (see table 13). In other regions of the nervous system, some neurons have a sequential requirement for combinations of neurotrophic factors (for example Hartnick et al. 1996), which could also be the case in the enteric nervous system.

A direct method to determine the influence of individual neurotrophic factors during postnatal development would be to study the enteric nervous system of conditional or inducible knock out mice that would exhibit loss of the neurotrophic factor of interest under specific conditions. Such mutant mice have proved useful in the study of neurotrophic factor functions in other regions of the nervous system (see Tessarollo 1998).

Mutant mice studies have revealed that during embryonic development GDNF is an absolute requirement for the survival of enteric precursors (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996). However it is not clear whether this trophic factor acts as a survival factor for postnatal enteric neurons and glial cells *in vivo* or *in vitro*. The change in proportions of these cells seen in the present study, while total cell numbers remained the same, is suggestive of an effect on precursor cells. More detailed studies in culture are needed to establish the precise nature of the effect on GDNF on neuronal numbers and whether enteric precursors are present in this culture system and *in vivo*.

GDNF was also found to stimulate an increase in neurite outgrowth from cultured myenteric neurons. Such an effect would be a useful way of ensuring increased neurite length and allowing contact with a growing target to be maintained. This would be important not only during growth, but also during remodelling that must occur as a result of mechanical stress exerted on myenteric ganglia by peristaltic contraction. Other neurotrophic factors could also be involved, such as NT-3 and CNTF which have also been shown to induce neurite length increases in culture (Saffrey et al. 1996; Silva et al. 1997 and see table 13). It has been suggested that due to its location, the enteric nervous system may require continued neurotrophic support in order to respond to potential damage sustained during peristaltic contractions (Gabella 1990) and also possible effects of gut toxins, such as bacteria in the gut especially in the large intestine. Breakdown in trophic support may lead to loss of neurons in ageing or acquired disease states.

In summary, the work presented in this thesis has highlighted the plasticity of the postnatal enteric nervous system and the importance of the postnatal period in establishing the adult pattern of innervation and diversity of phenotypes. Changes in myenteric neuronal numbers may be associated with changes in the smooth muscle, which is a target for many myenteric neurons.

Smooth muscle may be a source of trophic factors, such as GDNF which influence neuronal numbers and neurite outgrowth in the early postnatal period. The lack of a basal lamina may also allow direct contact between smooth muscle cells and ganglion cells and ready access to trophic factors, such as GDNF. Changes appear to centre around the weaning period which could suggest the involvement of diet-derived factors. A link has also been shown between diet and ageing in the enteric nervous system. Rats which were fed *ad libitum* did not live as long and exhibited decreased numbers of enteric neurons, when compared to rats maintained on a restricted diet (Feher 1992; Gabella 1989; Johnson et al. 1998). If dietary factors are shown to influence the postnatal development and maintenance of the enteric nervous, there could be implications for the regulation of diet, during the postnatal period and during ageing. Further study is needed to elucidate the factors involved in postnatal development and maintenance of the enteric nervous system and the long-term importance of diet in the maintenance of the enteric nervous system.

Future Directions

The results described in this thesis have raised several interesting questions regarding the postnatal development of the enteric nervous system and as a consequence, a large amount of work will need to be done in order to attempt to answer those questions. Examples of some of the issues that need to be addressed are described below;

- a) The total number of myenteric neurons increased between 7 and 14 days and again between 21 and 28 days. The source(s) of these “new” neurons remains to be identified and raises the question of whether uncommitted neural precursors are present in the enteric nervous system after the gut has become critical for survival.
- b) The *in vitro* study has revealed that in culture, treatment with GDNF leads to an increase in the number of neurons, without leading to an increase in the total number of cells, possibly suggesting the stimulation of uncommitted precursors to a

neuronal phenotype. It remains to be elucidated whether the same effect is observed *in vivo* and whether other signals are involved. Possible source(s) of endogenous GDNF and their relationship to myenteric neurons need to be characterised.

- c) Nitroergic neurons are among those that appear to be added to the myenteric plexus between 7 and 14 days, but not among those added between 21 and 28 days. The neurochemical identity of myenteric neurons that increase during these times need to be identified.
- d) A decrease was observed in the total number of myenteric neurons between 14 and 21 days. The phenotype of these neurons and their fate (i.e. migration to the submucous plexus or programmed cell death are two possibilities) need to be established.
- e) The ultrastructural study revealed that the number of myenteric ganglia surrounded by basal lamina increases during postnatal development. The study also revealed that a small proportion of myenteric ganglia remained in direct contact with surrounding tissue as they were not surrounded by a basal lamina. The functional significance of these findings needs to be investigated.
- f) The above changes were observed in development of the small intestine of the rat. It is important to know how the development of other regions of the gastrointestinal tract compares – is there a cranio-caudal gradient as observed for other aspects of vertebrate development?

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Appendix 1

Composition of Dulbecco's phosphate solution (dPBS)

Sodium chloride	8g/l
Potassium chloride	0.2g/l
Disodium hydrogen phosphate (Na_2HPO_4)	1.15g/l
Potassium di-hydrogen phosphate (KH_2PO_4)	0.2g/l
distilled water	Dissolve the above salts in 1 litre

Appendix 2

Composition of supplemented Hank’s balanced salt solution (HBSS) used in the in vitro study presented in chapter 6.

Component	Product Number	Final Concentration
HBSS X10	(Sigma, H1641)	X1.
Sodium Bicarbonate	(Sigma, S8761)	0.35g/l
Glucose	(Sigma, G7021)	5g/l
Sterile tissue culture grade water		846ml/l

Composition of supplemented balanced salt solution (BSS) used in the in vitro study presented in chapter 6.

Component	Product Number	Final Concentration
Modified HBBS Without calcium chloride or magnesium sulphate	(Sigma, H9394)	X1

Appendix 3

Composition of defined N1 culture media used in the *in vitro* study presented in chapter 6.

Component	Product Number	Final Concentration
199 medium	(Sigma, M7653)	X1
Glucose	(Sigma, G7021)	6g/l
Glutamine	(Sigma, G5763)	2mM
Sodium bicarbonate	(Sigma, S8761)	2g/l
Bovine serum albumin	(Sigma, A7638)	8g/l
Penicillin	(Sigma, P3414)	100U/ml
HEPES	(Sigma, H0887)	10mM
Insulin	(Sigma, I1882)	0.55U/ml
Transferrin	(Sigma, T0288)	0.5µg/l
Putrescence di-hydrochloride	(Sigma, P7505)	100µM
Sodium selenium	(Sigma, S1382)	$3 \times 10^{-8}M$
Progesterone	(Sigma, P0130)	$2 \times 10^{-8}M$
Tissue culture grade water		780ml/l